(54) Title: ANTIBODIES TO P-SELECTIN AND THEIR USES

(57) Abstract

The present invention relates to compositions and methods for treating inflammation and other pathological conditions using novel blocking P-selectin antibodies that inhibit adhesion of leukocytes to activated platelets and/or to activated vascular endothelium in vivo. Both murine and humanized antibodies are provided.

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ANTIBODIES TO P-SELECTIN AND THEIR USES

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of
08/057,292, filed May 5, 1993, which is a continuation-in-part
of 07/880,196, filed May 5, 1992, now abandoned. These
10 applications are incorporated by reference in their entirety
for all purposes.

TECHNICAL FIELD

This application relates generally to novel
15 immunoglobulins reactive with functional epitopes on a cell
surface receptor P-selectin. This application also relates
generally to diagnostic and therapeutic methods of using the
antibodies.

20 ### BACKGROUND OF THE INVENTION

The ability of cells to adhere to one another plays
a critical role in development, normal physiology, and disease
processes such as inflammation. This ability is mediated by
adhesion molecules, generally glycoproteins, expressed on cell
25 membranes. Often, an adhesion molecule on one cell type will
bind to another adhesion molecule expressed on a different
cell type, forming a receptor counter-receptor pair. Three
important classes of adhesion molecules are the integrins,
selectins, and immunoglobulin (Ig) superfamily members (*see*
30 Springer, *Nature* 346:425 (1990); Osborn, *Cell* 62:3 (1990);
Hynes, *Cell* 69:11 (1992), all of which are incorporated herein
by reference in their entirety for all purposes). These
molecules are especially vital to the interaction of
leukocytes and platelets with themselves and with the
35 extracellular matrix and vascular endothelium.

P-selectin, also known as CD62, granule membrane
protein-140 (GMP-140)/platelet activation-dependent granule
external membrane (PADGEM)/LECCAM-3 is a specialized cell-

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surface receptor on vascular endothelial cells and platelets
that is involved in the recognition of various circulating
cells. P-selectin is a surface glycoprotein with a lectin-
like domain, a region with homology to epidermal growth
5   factor, and a region with homology to complement regulatory
proteins (see McEver, *Blood Cells* 16:73-83 (1990)). The
structure of P-selectin is similar to that of two other
vascular cell surface receptors, endothelial leukocyte
adhesion molecule (ELAM-1) and lymphocyte homing receptor
10   (LHR). The term "selectin" has been suggested for this
general class of receptors, because of their lectin-like
domain and the selective nature of their adhesive functions.

P-selectin is present on the surface of platelets
and endothelial cells in response to a variety of stimuli,
15   where it mediates platelet-leukocyte and endothelium-leukocyte
interactions. By contrast, ELAM-1 is expressed only on
endothelial cells and LHR is expressed on a variety of
leukocytes in endothelial venules of peripheral lymph nodes.
Expression of P-selectin is inducible and it is not expressed
20   on unactivated endothelial cells or platelets. P-selectin
expression does not require *de novo* synthesis since it is
stored in secretory granules (or Weibel-Palade bodies) in both
platelets and endothelial cells. Thus, within minutes of
activation of either cell type by thrombin, histamine, or
25   phorbol esters or other humoral factors, P-selectin is rapidly
redistributed to the surface of the cell where it is
accessible for binding to other cells.

A number of mouse antibodies to P-selectin have been
reported (see, e.g., U.S. Patent No. 4,783,330, WO 91/06632,
30   PCT Application No. FR90/00565, McEver et al., *J. Biol. Chem.*
259:9799-9804 (1984)); Geng et al., *Nature* 343:757-760;
Parmentier et al., *Blood* 77:1734-1739 (1991)). An additional
antibody to P-selectin has been described in presentations at
the Scripps Institute, La Jolla California and at a Keystone
35   Colloquium held January 16, 1992. Certain of these antibodies
that block P-selectin mediated adhesion are Ca^{++} dependent.

In vitro experiments employing antibodies to P-
selectin have provided a limited understanding of the ligand

specificity of this receptor. It has been reported that P-selectin binds neutrophils, monocytes and certain carcinoma cells (Bevilacqua et al., *Proc. Natl. Acad. Sci. USA* 84:9238-9242 (1987); Geng et al., *Nature* 343:757-760 (1990); Larsen et al., *Cell* 63:467-474 (1990); and Polley et al. It has been also been reported that P-selectin recognizes Le^x as a high-affinity ligand (Larsen et al., *Cell* 63:467-474 (1990)). However, subsequent experiments showed that SLe^x-containing oligosaccharide is in fact a more potent inhibitor of P-selectin mediated adhesion than Le^x (Polley et al., *Proc. Natl. Acad. Sci. USA* 88:6224-6228 (1991)).

The participation of P-selectin in intercellular adhesion *in vitro* suggests that like other adhesion molecules, P-selectin might participate in cellular interactions that contribute to inflammatory diseases *in vivo*. However, the specific inflammatory diseases in which P-selectin might play a major role have not yet been elucidated. Similarly, the epitope-specificity of agents effective to abort particular P-selectin-mediated inflammatory diseases is also unclear. Of the known antibodies to P-selectin, some bind to P-selectin but do not block P-selectin mediated intercellular interactions. Other known antibodies have the capacity to block P-selectin-mediated intercellular binding only in the presence of high concentrations of Ca^{2+}, a condition that may not always exist *in vivo*. The absence of high concentrations of Ca^{2+} *in vivo* would render these antibodies ineffective as therapeutic agents. Moreover, all antibodies to P-selectin produced to-date have been of murine origin and might therefore induce a human-antimouse antibody response (HAMA) in clinical use.

Based on the foregoing it is apparent that a need exists for novel agents targeted against P-selectin that have the appropriate epitope-specificity to abort intercellular interactions leading to inflammatory diseases *in vivo*. Ideally such agents would not induce a HAMA response in treatment of humans. The present invention fulfills this and other needs.

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SUMMARY OF THE INVENTION

In one aspect of the invention, blocking antibodies that specifically bind to P-selectin are provided. One such exemplified antibody is designated mu MAb PB1.3 (produced by a
5 cell line having ATCC accession number HB11041). Other antibodies competitively inhibit the binding of mu MAb PB1.3 to P-selectin measured by a competitive inhibition assay. Preferred antibodies bind to P-selectin in the absence of Ca^{++}. The antibodies of the invention include fragments such
10 as Fab, Fab' F(ab')$_2$, Fabc, and Fv, as well as intact antibodies.

In another aspect of the invention, pharmaceutical compositions are provided. The pharmaceutical compositions comprise a blocking P-selectin antibody as described above and
15 a pharmaceutically acceptable carrier.

The invention also provides therapeutic methods for treating diseases of the immune system. The methods comprise administering to a patient having such a disease a therapeutically effective dose of a pharmaceutical composition
20 as described above. Acute lung injury and ischemic reperfusion injury are examples of diseases treatable by the present invention.

Some antibodies of the invention are humanized immunoglobulins. The humanized immunoglobulins comprise a
25 humanized heavy chain and a humanized light chain. The humanized light chain comprises three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of a mouse PB1.3 immunoglobulin light chain, and a
30 variable region framework from a human kappa light chain variable region framework sequence. The humanized heavy chain comprises three complementarity determining regions (CDR1, CDR2 and CDR3) having amino acid sequences from the corresponding complementarity determining regions of a mouse
35 PB1.3 immunoglobulin heavy chain, and a variable region framework from a human heavy chain variable region framework sequence. The humanized immunoglobulins specifically bind to P-selectin with a binding affinity having a lower limit of

about 10^7 M^{-1} and an upper limit of about five-times the binding affinity of the mouse PB1.3 immunoglobulin.

In many humanized immunoglobulins, the human kappa light chain variable region framework sequence is substituted in at least one position selected from a first group consisting of L21, L46, L60 and L70, by an amino acid present in the equivalent position of the mouse PB1.3 immunoglobulin light chain variable region framework sequence. Similarly, the human heavy chain variable region framework sequence is substituted in at least one position selected from a second group consisting of H1, H2, H71 and H73, by an amino acid present in the equivalent position of the mouse PB1.3 heavy chain variable region framework sequence. In some humanized immunoglobulins, the humanized heavy chain variable region framework is a 21/28'CL heavy chain variable region framework sequence. In some humanized immunoglobulins, the humanized light chain variable region framework is a DEN light chain variable region framework sequence. Exemplary mature light chain variable region amino acid sequences are shown in Figures 14-17. Exemplary mature heavy chain variable region amino acid sequences are shown in Figures 11-13. A preferred humanized immunoglobulin comprises the mature light chain variable region shown in Figure 17 and the mature heavy chain variable region shown in Figure 12.

The invention provides intact humanized immunoglobulins and humanized immunoglobulin fragments such as Fab, Fab' F(ab')$_2$, Fabc and Fv. Some humanized immunoglobulins further comprise a constant region, which may or may not have an effector function.

In another aspect of the invention, nucleic acids encoding the humanized immunoglobulins described above are provided. Some nucleic acid encode heavy chains and others encode light chains.

The invention also provides computers programmed to display a three dimensional representation of the immunoglobulins described above on a monitor or printer.

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The invention further provides pharmaceutical compositions comprising the humanized immunoglobulins described above and a pharmaceutically acceptable carrier.

In another aspect, the invention provides methods for detecting P-selectin using the humanized immunoglobulins described above. A humanized immunoglobulin is administered to a patient or a tissue sample therefrom. Complexes formed by specific binding between the immunoglobulin and P-selectin present in the target sample are detected to indicate the presence of P-selectin.

The invention also provides methods of treating a patient suffering from a disease of the immune systems using humanized immunoglobulins. The methods comprise administering to the patient a therapeutically effective dose of a pharmaceutical composition as described above. Diseases amenable to treatment include ischemia-reperfusion injury and acute lung injury. For example, the compositions are useful for treating patients suffering from epidermal, myocardial, renal, cerebral, splenic, hepatic, spinal, splanchnic, pulmonary, partial-body, or whole-body ischemia.

In a further aspect of the invention, stable cell lines producing humanized immunoglobulins are provided. The stable cell lines comprise two nucleic acids segments. One nucleic acid segment encodes the heavy chain of a humanized immunoglobulin as described above, the segment operably linked to a promoter to allow expression of the heavy chain. The second nucleic acid segment encodes the light chain of the humanized immunoglobulin, the second segment operably linked to a second promoter to allow expression of the light chain. Preferred cell lines produce about 30 μg of the humanized immunoglobulin/10^6 cells/day.

In another aspect of the invention, fragments of P-selectin are provided. These fragments have up to 100 amino acids, the up to 100 amino acids comprising at least five contiguous amino acids from between positions 448 and 467 of the amino sequence shown in Fig. 34. In preferred fragments, the up to 100 amino acids comprise the entire contiguous segment of amino acids between positions 448 and 467.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 presents data showing that anti-inflammatory immunoglobulins of the invention inhibit binding of thrombin activated platelets to neutrophils.

5   Figs. 2A and 2B show that anti-inflammatory immunoglobulins of the invention effectively prevent lung injury induced by infusion of cobra venom factor (CVF) as measured by permeability (Fig. 2A) and hemorrhage (Fig 2B).

Fig. 3A through 3H show that P-selectin expression

10   content in lungs of animals is upregulated in response to cobra venom infusion visualized by horseradish proxidase stain of mu MAb PB1.3 panel (a)-(d) show expression of P-selectin in lung venules at various times after infusion of CVF; time 0 (a); 5, 10 and 15 min, panels b, c and d, respectively (Fig.

15   3A). Fig. 3B are light and transmission electron micrographs of CVF-induced acute lung injury in rats treated with 200 μg non-blocking antibody (mu MAb PNB1.6) (frames a.c) or with blocking P-selectin antibody (mu MAb PB1.3) to P-selectin (frames b.d). In animals treated with PNB1.6, vascular injury

20   was indicated by extensive intra-alveolar hemorrhage (frame a), associated with intravascular aggregates of neutrophils (frame c. arrows) in close contrast with endothelial cells. In contrast, in animals treated with mu MAb PB1.3 intra-alveolar hemorrhage was absent (frame b) and intravascular

25   neutrophils showed little contact with the endothelium (frame d. arrows). (Frames a and b, plastic embedded sections stained with toluidine blue, X160: frames c and d, uranyl acetate, lead citrate stained X2250.)

Fig. 4 shows the binding of the monoclonal antibody

30   mu MAb PB1.3 to P-selectin: effect of chelating divalent cations with EDTA. In the plot the open symbols (lower trace) is binding in the presence of Ca^{++} and Mg^{++}, while the filled symbols (upper trace) is binding in the presence of EDTA. Antibody was diluted shown at 1.6 mg/ml.

35   Fig. 5 shows the effect of the peptide CQNRYTDLVAIQNKNE on the binding of mu MAb PB1.3 to P-selectin. Filled symbols are in the presence of 0.35 mg/ml peptide, open

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symbols are in the absence of peptides.   Indicated dilutions
of mu MAb PB1.3 were from a 1.6 mg/ml solution.

Figs. 6A, 6B and 6C show the ability of monoclonal
antibodies to cross-block binding to P-selectin. Blocking
5   antibodies were present at 50 μg/ml. Dilutions of
biotinylated antibodies shown were from 1.6 mg/ml mu MAb
PB1.3, 0.87 mu MAb mg/ml PNB1.6, and 0.59 mg/ml mu MAb 84/26.

Fig. 7 shows that the extent of platelet aggregation
induced by thrombin is equivalent under control conditions in
10   the absence of antibody and in the presence of increasing
concentrations (1-100 μg/ml) of purified mu MAb PB1.3
antibody.

Fig. 8 shows that the extent of myocardial ischemia,
expressed as a percentage of the area at risk, is
15   significantly less in animals treated with the blocking anti-
P-selectin antibody (mu MAb PB1.3) than in animals treated
with the non-blocking anti-P-selectin antibody. Furthermore,
the ability of coronary artery rings to relax in response to
acetylcholine is significantly greater in mu MAb PB1.3
20   compared with mu MAb PNB1.6 treated animals. That these
phenomena are attributable to a reduction in myocardial
leukocyte infiltration is suggested by the reduced number of
PMNs in myocardial tissue from mu MAb PB1.3 treated animals
compared with PNB1.6 treated animals.

25   Fig. 9 shows the sequence and translation of PB1.3
heavy chain signal peptide and variable region.

Fig. 10 shows the sequence and translation of PB1.3
light chain signal peptide and variable region.

Fig. 11 shows the sequence and translation of CY1748
30   heavy chain-A signal peptide and variable region.

Fig. 12 shows the sequence and translation of CY1748
heavy chain-B signal peptide and variable region.

Fig. 13 shows the sequence and translation of CY1748
heavy chain-C signal peptide and variable region.

35   Fig. 14 shows the sequence and translation of CY1748
light chain-A signal peptide and variable region.

Fig. 15 shows the sequence and translation of CY1748
light chain-B signal peptide and variable region.

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Fig. 16 shows the sequence and translation of CY1748 light chain-C signal peptide and variable region.

Fig. 17 shows the sequence and translation of CY1748 light chain-D signal peptide and variable region.

Fig. 18 shows physical map of the neo-plasmid expressing the human heavy chain (gamma-1 constant region).

Fig. 19 shows the physical map of the neo-plasmid expressing the human light chain (kappa constant region).

Fig. 20 shows the binding curves of the chimeric PB1.3 and reshaped versions of CY1748 to rsP-Selectin.

Fig. 21 shows the binding curves of the chimeric PB1.3 and reshaped versions of CY1748 to rsP-Selectin.

Fig. 22 shows the binding curves of the chimeric PB1.3 and reshaped versions of CY1748 to rsP-Selectin.

Fig. 23 shows the physical map of the dhfr-plasmid expressing the human heavy chain (gamma-1 constant region).

Fig. 24 shows the physical map of the dhfr-plasmid expressing the human heavy chain (gamma-4 constant region).

Fig. 25 shows the inhibition of biotinylated-PB1.3 binding to rsP-Selectin by PB1.3 and CY1748 RH_A/RL_A-IgG1.

Fig. 26 shows the inhibition of biotinylated-PB1.3 binding to rsP-Selectin by PB1.3 and CY1748 RH_B/RL_D-IgG4.

Fig. 27 shows that antibody PB1.3 reduces swelling associated with ischemia and reperfusion in the rabbit ear.

Fig. 28 shows that antibody PB1.3 prevents the development of necrosis following ischemia and reperfusion of the rabbit ear.

Fig. 29 shows that antibody PB1.3 inhibits leukocyte-endothelial cell interactions induced by the mast cell degranulating agent compound 48/80.

Fig. 30 shows the proposed folding pattern of the domains of P-selectin.

Fig. 31 shows a schematic of the deletion mutants of P-selectin used to map the epitope that PB1.3 recognizes.

Fig. 32 shows the binding of PB1.3 and rabbit polyclonal antibodies to P-selectin deletion mutants.

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Fig. 33 shows the PB1.3 and P6H6 binding to P-selectin and to P-selectin Δ5CRP which is a deletion mutant of P-selectin that lacks the fifth CRP.

Fig. 34 shows the sequences of synthetic peptides
5   derived from the amino acid sequence of the human P-selectin 5th CRP and the PB1.3 reactivity to these immobilized peptides. Amino acids are numbered according to the convention of Johnston et al., *Cell*, 56:1033-1044 (1989) for a full-length P-selectin polypeptide.

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DEFINITIONS

Abbreviations for the twenty naturally occurring amino acids follow conventional usage (*Immunology - A Synthesis* (2nd ed., E.S. Golub & D.R. Gren, eds., Sinauer
15   Associates, Sunderland, MA, 1991)). Stereoisomers (*e.g.*, D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the
20   present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and other similar amino
25   acids and imino acids (*e.g.*, 4-hydroxyproline). Moreover, amino acids may be modified by glycosylation, phosphorylation and the like.

In the polypeptide notation used herein, the lefthand direction is the amino terminal direction and the
30   righthand direction is the carboxy-terminal direction, in accordance with standard usage and convention. Similarly, unless specified otherwise, the lefthand end of single-stranded polynucleotide sequences is the 5' end; the lefthand direction of double-stranded polynucleotide sequences is
35   referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5'

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end of the RNA transcript are referred to as "upstream
sequences"; sequence regions on the DNA strand having the same
sequence as the RNA and which are 3' to the 3' end of the RNA
transcript are referred to as "downstream sequences".

5   The phrase "polynucleotide sequence" refers to a
single or double-stranded polymer of deoxyribonucleotide or
ribonucleotide bases read from the 5' to the 3' end. It
includes self-replicating plasmids, infectious polymers of DNA
or RNA and non-functional DNA or RNA.

10   The following terms are used to describe the
sequence relationships between two or more polynucleotides:
"reference sequence", "comparison window", "sequence
identity", "percentage of sequence identity", and "substantial
identity". A "reference sequence" is a defined sequence used
15 as a basis for a sequence comparison; a reference sequence may
be a subset of a larger sequence, for example, as a segment of
a full-length cDNA or gene sequence given in a sequence
listing, such as a polynucleotide sequence of Figs. 9-17, or
may comprise a complete DNA or gene sequence. Generally, a
20 reference sequence is at least 20 nucleotides in length,
frequently at least 25 nucleotides in length, and often at
least 50 nucleotides in length. Since two polynucleotides may
each (1) comprise a sequence (*i.e.*, a portion of the complete
polynucleotide sequence) that is similar between the two
25 polynucleotides, and (2) may further comprise a sequence that
is divergent between the two polynucleotides, sequence
comparisons between two (or more) polynucleotides are
typically performed by comparing sequences of the two
polynucleotides over a "comparison window" to identify and
30 compare local regions of sequence similarity. A "comparison
window", as used herein, refers to a conceptual segment of at
least 20 contiguous nucleotide positions wherein a
polynucleotide sequence may be compared to a reference
sequence of at least 20 contiguous nucleotides and wherein the
35 portion of the polynucleotide sequence in the comparison
window may comprise additions or deletions (*i.e.*, gaps) of 20
percent or less as compared to the reference sequence (which
does not comprise additions or deletions) for optimal

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alignment of the two sequences. Optimal alignment of
sequences for aligning a comparison window may be conducted by
the local homology algorithm of Smith & Waterman, *Adv. Appl.*
Math. 2:482 (1981), by the homology alignment algorithm of

5   Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search
for similarity method of Pearson & Lipman, *Proc. Natl. Acad.*
Sci. (USA) 85:2444 (1988) (each of which is incorporated by
reference in its entirety for all purposes), by computerized
implementations of these algorithms (GAP, BESTFIT, FASTA, and

10 TFASTA in the Wisconsin Genetics Software Package Release 7.0,
Genetics Computer Group, 575 Science Dr., Madison, WI), or by
inspection, and the best alignment (*i.e.,* resulting in the
highest percentage of sequence similarity over the comparison
window) generated by the various methods is selected. The

15 term "sequence identity" means that two polynucleotide
sequences are identical (*i.e.,* on a nucleotide-by-nucleotide
basis) over the window of comparison. The term "percentage of
sequence identity" is calculated by comparing two optimally
aligned sequences over the window of comparison, determining

20 the number of positions at which the identical nucleic acid
base (*e.g.,* A, T, C, G, U, or I) occurs in both sequences to
yield the number of matched positions, dividing the number of
matched positions by the total number of positions in the
window of comparison (*i.e.,* the window size), and multiplying

25 the result by 100 to yield the percentage of sequence
identity. The terms "substantial identity" as used herein
denotes a characteristic of a polynucleotide sequence, wherein
the polynucleotide comprises a sequence that has at least 85
percent sequence identity, preferably at least 90 to 95

30 percent sequence identity, more usually at least 99 percent
sequence identity as compared to a reference sequence over a
comparison window of at least 20 nucleotide positions,
frequently over a window of at least 25-50 nucleotides,
wherein the percentage of sequence identity is calculated by

35 comparing the reference sequence to the polynucleotide
sequence which may include deletions or additions which total
20 percent or less of the reference sequence over the window
of comparison. The reference sequence may be a subset of a

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larger sequence, for example, the sequence shown in Figs. 10-17.

As applied to polypeptides, the term "sequence identity" means peptides share identical amino acids at
5 corresponding positions. The term "sequence similarity" means peptides have identical or similar amino acids (*i.e.*, conservative substitutions) at corresponding positions. The term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT
10 using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (*e.g.*, 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino
15 acid substitutions. The term "substantial similarity" means that two peptide sequences share corresponding percentages of sequence similarity.

The term "substantially pure" means an object species is the predominant species present (*i.e.*, on a molar
20 basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially
25 pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods)
30 wherein the composition consists essentially of a single macromolecular species.

For purposes of classifying amino acids substitutions as conservative or nonconservative, amino acids are grouped as follows: Group I (hydrophobic sidechains):
35 norleucine, met, ala, val, leu, ile; Group II (neutral hydrophilic side chains): cys, ser, thr; Group III (acidic side chains): asp, glu; Group IV (basic side chains): asn, gln, his, lys, arg; Group V (residues influencing chain

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orientation): gly, pro; and Group VI (aromatic side chains):
trp, tyr, phe. Conservative substitutions involve
substitutions between amino acids in the same class. Non-
conservative substitutions constitute exchanging a member of
5   one of these classes for another.

        Amino acids from the variable regions of the mature
heavy and light chains of immunoglobulins are designated Hx
and Lxx respectively, where x is a number designating the
position of an amino acids according to the scheme of Kabat et
10   al., *Sequences of Proteins of Immunological Interest* (National
Institutes of Health, Bethesda, MD (1987) and (1991))
(hereinafter collectively referred to as "Kabat et al.,"
incorporated by reference in their entirety for all purposes).
Kabat et al. list many amino acid sequences for antibodies for
15   each subclass, and list the most commonly occurring amino acid
for each residue position in that subclass. Kabat et al. use
a method for assigning a residue number to each amino acid in
a listed sequence, and this method for assigning residue
numbers has become standard in the field. Kabat et al.'s
20   scheme is extendible to other antibodies not included in the
compendium by aligning the antibody in question with one of
the consensus sequences in Kabat et al. The use of the Kabat
et al. numbering system readily identifies amino acids at
equivalent positions in different antibodies. For example, an
25   amino acid at the L50 position of a human antibody occupies
the equivalence position to an amino acid position L50 of a
mouse antibody.

        "Immunoglobulin," "antibody" or "antibody
peptide(s)" refers to an intact antibody or a binding fragment
30   thereof that competes with the intact antibody for specific
binding to P-selectin.

        "Substantial inhibition" means at least about 50%
inhibition, preferably about 60% to about 80%, and more
usually about greater than 85% or more (as measured in an *in
35   vitro* competitive binding assay).

        Chimeric antibodies are antibodies whose light and
heavy chain genes have been constructed, typically by genetic
engineering, from immunoglobulin gene segments belonging to

different species. For example, the variable (V) segments of
the genes from a mouse monoclonal antibody may be joined to
human constant (C) segments, such as γ_1 and γ_4. A typical
therapeutic chimeric antibody is thus a hybrid protein
consisting of the V or antigen-binding domain from a mouse
antibody and the C or effector domain from a human antibody,
although other mammalian species may be used.

DESCRIPTION OF THE PREFERRED EMBODIMENT

This invention provides compositions and methods for
inhibiting diseases and conditions of the immune system
mediated by P-selectin. Specifically, the invention provides
immunoglobulins which have the ability to inhibit P-selectin-
mediated adhesion of cells *in vivo*.

I. Characteristics of Immunoglobulins

A. General

The basic antibody structural unit is known to
comprise a tetramer. Each tetramer is composed of two iden-
tical pairs of polypeptide chains, each pair having one
"light" (about 25 kDa) and one "heavy" chain (about 50-70
kDa). The amino-terminal portion of each chain includes a
variable region of about 100 to 110 or more amino acids
primarily responsible for antigen recognition. The carboxy-
terminal portion of each chain defines a constant region
primarily responsible for effector function.

Light chains are classified as either kappa or
lambda. Heavy chains are classified as gamma, mu, alpha,
delta, or epsilon, and define the antibody's isotype as IgG,
IgM, IgA, IgD and IgE, respectively. Within light and heavy
chains, the variable and constant regions are joined by a "J"
region of about 12 or more amino acids, with the heavy chain
also including a "D" region of about 10 more amino acids.
(*See generally, Fundamental Immunology* (Paul, W., ed., 2nd ed.
Raven Press, N.Y., 1989), Ch. 7 (incorporated by reference in
its entirety for all purposes).

The variable regions of each light/heavy chain pair
form the antibody binding site. The chains all exhibit the

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same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair are aligned by the framework
5   regions, enabling binding to a specific epitope. CDR and FR residues are delineated according to the standard sequence definition of Kabat et al., *supra*. An alternative structural definition has been proposed by Chothia et al., *J. Mol. Biol.* 196:901-917 (1987); *Nature* 342:878-883 (1989); and *J. Mol.*
10   *Biol.* 186:651-663 (1989) (hereinafter collectively referred to as "Chothia et al." and incorporated by reference in their entirety for all purposes).

          B.   Binding Specificity and Affinity
15   The immunoglobulins (or antibodies) of the invention selectively bind a functional epitope on P-selectin associated with a response to tissue injury and inflammation. Usually, binding of the antibodies to a functional epitope on P-selectin effectively inhibits adhesion of leukocytes to
20   activated platelets and/or to the activated vascular endothelium *in vivo*. Antibodies showing this property are referred to as "blocking" antibodies. Preferred blocking antibodies impair the adhesion of leukocytes to the activated vascular endothelium to prevent or inhibit an inflammatory
25   and/or thrombotic condition.
           Many of the blocking antibodies of the invention compete with an exemplary antibody designated mu MAb PB1.3 for specific binding to P-selectin. A hybridoma producing the mu MAb PB1.3 antibody has been deposited with the American Type
30   Culture Collection, Rockville, Maryland under the Budapest Treaty on ___ and given the Accession No. HB11041. The production of this monoclonal antibody is described in Example 1. Other blocking antibodies of the invention compete with a second exemplified antibody designated 84/26.
35   Competition is determined by an assay in which the immunoglobulin under test inhibits specific binding of a reference antibody (e.g., mu MAb PB1.3) to an antigenic determinant on a P-selectin molecule. Numerous types of

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competitive binding assays are known for example: solid phase
direct or indirect radioimmunoassay (RIA), solid phase direct
or indirect enzyme immunoassay (EIA), sandwich competition
assay (see Stahli et al., *Methods in Enzymology* 9:242-253

5   (1983)); solid phase direct biotin-avidin EIA (see Kirkland et
al., *J. Immunol.* 137:3614-3619 (1986)); solid phase direct
labeled assay, solid phase direct labeled sandwich assay (see
Harlow and Lane, "Antibodies, A Laboratory Manual," Cold
Spring Harbor Press (1988)); solid phase direct label RIA

10   using I-125 label (see Morel et al., *Molec. Immunol.* 25(1):7-
15 (1988)); solid phase direct biotin-avidin EIA (Cheung et
al., *Virology* 176:546-552 (1990)); and direct labeled RIA
(Moldenhauer et al., *Scand. J. Immunol.* 32:77-82 (1990)).
Typically, such an assay involves the use of purified P-

15   selectin or cells bearing P-selectin bound to a solid surface,
an unlabelled test immunoglobulin and a labelled reference
immunoglobulin. Competitive inhibition is measured by
determining the amount of label bound to the solid surface in
the presence of the test immunoglobulin. Usually the test

20   immunoglobulin is present in excess. An example of a suitable
competitive binding assay is presented in Example 8.
Antibodies identified by competition assay (competing
antibodies) include antibodies binding to the same epitope as
the reference antibody and antibodies binding to an adjacent

25   epitope sufficiently proximal to the epitope bound by the
reference antibody for steric hindrance to occur. Usually,
when a competing antibody is present in excess, it will
inhibit specific binding of a reference antibody to P-selectin
by at least 10, 25, 50 or 75%.

30   The epitope bound by mu MAb PB1.3 has been mapped to
an epitope between amino acids 448 and 467 of human P-
selectin. *See* Figure 34 and Johnson et al., *Cell* 56:1033-1044
(1989) (hereby incorporated by reference in its entirety for
all purposes). The epitope occurs within the fifth CRP domain

35   of P-selectin. *See* Figure 30. Antibodies that compete with
mu MAb PB1.3 usually bind to a segment of amino acids between
448 and 467 or to another segment within the fifth CRP domain.

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The binding specificity of many blocking antibodies
of the invention is further defined by their capacity to bind
P-selectin in the complete or substantial absence of Ca^{++}
(e.g., in the presence of 25 mM EDTA (a calcium chelator) and
5   the absence of Ca^{++} in an in vitro assay). Antibodies
requiring a Ca^{++} cofactor for blocking activity may not be
effective in in vivo conditions where levels of Ca^{++} are
expected to fluctuate.

Antibodies that compete with the mu MAb PB1.3
10 antibody are further characterized by the observation that
their capacity to specifically bind to P-selectin is not
inhibited by a fragment of P-selectin having the amino acid
sequence CQNRYTDLVAIQNKNE. A molar excess of this fragment
usually inhibits specific binding of such antibodies by less
15 than 5%. Mu MAb PB1.3 and competing antibodies are thereby
distinguished from reported mouse antibodies designated G1, G2
and G3 (Geng et al., The Journal of Biological Chemistry
266:22313-22318 (1991)) whose binding to P-selectin is
partially or completely blocked by the above peptide.

20   Some antibodies of the invention are further
characterized by their capacity to selectively inhibit some P-
selectin mediated responses while leaving others unimpaired.
For example, some antibodies are capable of blocking specific
binding of neutrophils to activated endothelial cells in vivo,
25 without inhibiting platelet aggregation. Such antibodies are
particularly useful for treating inflammatory disorders
without impairing a patient's capacity to initiate a response
to conditions (e.g., wounding) in which platelet aggregation
is desirable.

30   The antibodies of the invention usually exhibit a
specific binding affinity for P-selectin of at least 10^7, 10^8,
10^9, or 10^{10} M^{-1}. Usually the upper limit of binding affinity
of the antibodies for P-selectin is within a factor of about
three, five or ten of that of the mu MAb PB 1.3. Often the
35 lower limit of binding affinity is also within a factor of
about three, five or ten of that of mu MAb PB1.3. In this
context, the term "about" encompasses the small degree of

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experimental error that may typically occur in the measurement of binding affinities.

II.   Production of Immunoglobulins

A.   Nonhuman Antibodies

5   The production of non-human monoclonal antibodies, e.g., murine, lagomorpha, equine is well known and can be accomplished by, for example, immunizing an animal with a preparation containing cells bearing P-selectin (e.g., thrombin-activated platelets), isolated P-selectin molecules

10   or fragments thereof, such as extracellular domains. Antibody-producing cells obtained from the immunized animals are immortalized and screened, or screened first for the production of antibody which binds to P-selectin, and then immortalized. *See* Harlow and Lane, *supra*. Alternatively,

15   substantially monospecific antibody populations can be produced by chromatographic purification of polyclonal sera.

B.   Human Antibodies Against P-Selectin

In another aspect of the invention, human antibodies

20   against P-selectin are provided. Some human antibodies are selected by competitive binding experiments, or otherwise, to have the same epitope specificity as a particular mouse antibody, such as mu MAb PB1.3. Such antibodies are particularly likely to share the useful therapeutic properties

25   demonstrated for mu MAb PB1.3. Human antibodies to P-selectin can be produced by screening a DNA library from human B cells according to the general protocol outlined by Huse et al., *Science* 246:1275-1281 (1989). Antibodies binding to P-selectin or a fragment thereof are selected. Sequences

30   encoding such antibodies (or a binding fragments) are then cloned and amplified. The protocol described by Huse is rendered more efficient in combination with phage-display technology. *See, e.g.,* Dower et al., WO 91/17271 and McCafferty et al., WO 92/01047 (each of which is incorporated

35   by reference in its entirety for all purposes). In these methods, libraries of phage are produced in which members display different antibodies on their outersurfaces. Antibodies are usually displayed as Fv or Fab fragments.

20

Phage displaying antibodies with a desired specificity are
selected by affinity enrichment to an P-selectin polypeptide
or fragment thereof.

        In a variation of the phage-display method, human
5   antibodies having the binding specificity of a selected murine
    antibody can be produced. *See* Winter, WO 92/20791. In this
    method, either the heavy or light chain variable region of the
    selected murine antibody (*e.g.*, mu MAb PB1.3) is used as a
    starting material. If, for example, a light chain variable
10 region is selected as the starting material, a phage library
    is constructed in which members displays the same light chain
    variable region (*i.e.*, the murine starting material) and a
    different heavy chain variable region. The heavy chain
    variable regions are obtained from a library of rearranged
15 human heavy chain variable regions. A phage showing strong
    specific binding for P-selectin (*e.g.*, at least 10^8 and
    preferably at least 10^9 M^{-1}) is selected. The human heavy
    chain variable region from this phage then serves as a
    starting material for constructing a further phage library.
20 In this library, each phage displays the same heavy chain
    variable region (*i.e.*, the region identified from the first
    display library) and a different light chain variable region.
    The light chain variable regions are obtained from a library
    of rearranged human variable light chain regions. Again,
25 phage showing strong specific binding for L-selectin are
    selected. These phage display the variable regions of
    completely human anti-P-selectin antibodies. These antibodies
    usually have the same or similar epitope specificity as the
    murine starting material (*e.g.*, mu MAb PB1.3).

30

        C. Humanized Antibodies
        The invention provides humanized immunoglobulins
    having variable framework regions substantially from a human
    immunoglobulin (termed an acceptor immunoglobulin) and
35 complementarity determining regions substantially from a mouse
    immunoglobulin termed mu MAb PB1.3 (referred to as the donor
    immunoglobulin). The constant region(s), if present, are also
    substantially from a human immunoglobulin. The humanized

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antibodies of the present invention offer several advantages over the mouse donor antibody, which has already shown to be effective in animals models:

     1)   The human immune system should not recognize
5   the framework or constant region of the humanized antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody or a partially foreign chimeric antibody.

10   2)   Because the effector portion of the humanized antibody is human, it may interact better with other parts of the human immune system.

     3)   Injected mouse antibodies have been reported to have a half-life in the human circulation much shorter than
15   the half-life of normal human antibodies (Shaw et al., *J. Immunol.* 138:4534-4538 (1987)). Injected humanized antibodies have a half-life essentially equivalent to naturally occurring human antibodies, allowing smaller and less frequent doses.

20   1.   Cloning and Sequencing Variable Domains of mu MAb PB1.3

     The cloning and sequencing of cDNA encoding the mu MAb PB1.3 antibody heavy and light chain variable regions is described in Example 10, and the nucleotide and predicted
25   amino acids sequences are shown in Figs. 11 and 12. Tables 1 and 2 illustrate the subdivision of the amino acid coding sequence into framework and complementarity determining domains. From N-terminal to C-terminal, both light and heavy chains comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3
30   and FR4. The assignment of amino acids to each domain is in accordance with the numbering convention of Kabat et al., *supra*.

     (2)   Selection of Human Antibodies to Supply
35 Framework Residues

     The substitution of mouse CDRs into a human variable domain framework is most likely to result in retention of their correct spatial orientation if the human variable domain

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framework adopts the same or similar conformation to the mouse
variable framework from which the CDRs originated. This is
achieved by obtaining the human variable domains from human
antibodies whose framework sequences exhibit a high degree of
5   sequence identity with the murine variable framework domains
from which the CDRs were derived. The heavy and light chain
variable framework regions can be derived from the same or
different human antibody sequences. The human antibody
sequences can be the sequences of naturally occurring human
10   antibodies or can be consensus sequences of several human
antibodies. See Kettleborough et al., *Protein Engineering*
4:773 (1991); Kolbinger et al., *Protein Engineering* 6:971
(1993).

Suitable human antibody sequences are identified by
15   computer comparisons of the amino acid sequences of the mouse
variable regions with the sequences of known human antibodies.
The comparison is performed separately for heavy and light
chains but the principles are similar for each. This
comparison reveals that the mu MAb PB1.3 light chain shows
20   greatest sequence identity to human light chains of subtype
kappa-1 and that the mu PB1.3 heavy chain shows greatest
sequence identity to human heavy chains of subtype 1, as
defined by Kabat et al., *supra*. Thus, light and heavy human
framework regions are usually derived from human antibodies of
25   these subtypes, or from consensus sequences of such subtypes.
The preferred heavy and light chain human variable regions
showing greatest sequence identity to the corresponding
regions from mu MAb PB1.3 are from antibodies 21/28'CL and DEN
respectively.

30

(3)   Computer Modelling

The unnatural juxtaposition of murine CDR regions
with human variable framework region can result in unnatural
conformational restraints, which, unless corrected by
35   substitution of certain amino acid residues, lead to loss of
binding affinity. The selection of amino acid residues for
substitution is determined, in part, by computer modelling.
Computer hardware and software for producing three-dimensional

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images of immunoglobulin molecules are widely available. In
general, molecular models are produced starting from solved
structures for immunoglobulin chains or domains thereof. The
chains to be modelled are compared for amino acid sequence
5   similarity with chains or domains of solved three dimensional
structures, and the chains or domains showing the greatest
sequence similarity is/are selected as starting points for
construction of the molecular model. For example, for the
light chain of mu MAb PB1.3, the starting point for modelling
10   was the human light chain DEN. The solved starting structures
are modified to allow for differences between the actual amino
acids in the immunoglobulin chains or domains being modelled,
and those in the starting structure. The modified structures
are then assembled into a composite immunoglobulin. Finally,
15   the model is refined by energy minimization and by verifying
that all atoms are within appropriate distances from one
another and that bond lengths and angles are within chemically
acceptable limits. Such a model can in turn serve as a
starting point for predicting the three-dimensional structure
20   of an antibody containing the mu MAb PB1.3 complementarity
determining regions substituted in human framework structures.
Additional models can be constructed representing the
structure when further amino acid substitutions to be
discussed *infra*, are introduced.
25

(4)   Substitution of Amino Acid Residues

As noted *supra*, the humanized antibodies of the
invention comprise variable framework regions substantially
from a human immunoglobulin and complementarity determining
30   regions substantially from a mouse immunoglobulin termed mu
MAb PB1.3. Having identified the complementarity determining
regions of mu MAb PB1.3 and appropriate human acceptor
immunoglobulins, the next step is to determine which, if any,
residues from these components should be substituted to
35   optimize the properties of the resulting humanized antibody.
In general, substitution of human amino acid residues with
murine should be minimized, because introduction of murine
residues increases the risk of the antibody eliciting a HAMA

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response in humans. Amino acids are selected for substitution
based on their possible influence on CDR conformation and/or
binding to antigen. Investigation of such possible influences
is by modelling, examination of the characteristics of the
amino acids at particular locations, or empirical observation
of the effects of substitution or mutagenesis of particular
amino acids.

When an amino acid differs between a mu MAb PB1.3
variable framework region and an equivalent human variable
framework region, the human framework amino acid should
usually be substituted by the equivalent mouse amino acid if
it is reasonably expected that the amino acid:

(1) noncovalently binds antigen directly (*e.g.*,
amino acids at positions H1, H2, and L60 of mu MAb PB1.3)

(2) is adjacent to a CDR region, is part of a CDR
region under the alternative definition proposed by Chothia et
al., *supra*, or otherwise interacts with a CDR region (*e.g.*, is
within about 3 Å of a CDR region) (*e.g.*, H73 of mu MAb PB1.3,
Tables 1 and 3), or

(3) participates in the V_L-V_H interface.

Other candidates for substitution are acceptor human
framework amino acids that are unusual for a human
immunoglobulin at that position. These amino acids can be
substituted with amino acids from the equivalent position of
more typical human immunoglobulins. Alternatively, amino
acids from equivalent positions in mu MAb PB1.3 can be
introduced into the human framework regions when such amino
acids are typical of human immunoglobulin at the equivalent
positions.

In general, substitution of all or most of the amino
acids fulfilling the above criteria is desirable.
Occasionally, however, there is some ambiguity about whether a
particular amino acid meets the above criteria, and
alternative variant immunoglobulins are produced, one of which
has that particular substitution, the other of which does not.
Surprisingly, an exemplified humanized antibody designated
reshaped MAb PB1.3 ($H_B L_D$) contains only one substitution of
the human heavy-chain variable region framework residues and

25

only two substitutions of the human light-chain variable
region framework residues and yet exhibits substantially
similar (within a factor of two) binding affinity to a murine
or chimeric antibody having intact murine variable regions
5   fused to a human constant region (designated chi MAb PB1.3).
       Some humanized antibodies of the invention contain a
substitution of a human light chain framework residue with a
corresponding mu MAb PB1.3 residue in at least 1, 2 or 3, and
sometimes 4 of the following positions: L21, L46, L60 and L70.
10   (See Tables 2 and 4). Some humanized antibodies usually
contain a substitution of a human heavy chain framework
residue in at least 1, 2 or 3 and sometimes 4 of the following
positions H1, H2, and H71 and H73. (See Tables 1 and 3) Many
humanized antibodies contain substitutions of both light and
15   heavy chain variable framework regions. Preferred antibodies
contain a substitution of a human heavy chain framework
residue at least at position H71, and substitutions of human
light chain framework residues at least at positions 21 and
46.
20   Usually the CDR regions in humanized antibodies are
substantially identical, and more usually, identical to the
corresponding CDR regions in the mu MAb PB1.3 antibody.
Although not usually desirable, it is sometimes possible to
make one or more conservative amino acid substitutions of CDR
25   residues without appreciably affecting the binding affinity of
the resulting humanized immunoglobulin. Occasionally,
substitution of CDR regions can result in enhanced binding
affinity.
       Other than for the specific amino acid substitutions
30   discussed above, the framework regions of humanized
immunoglobulins are usually substantially identical, and more
usually, identical to the framework regions of the human
antibodies from which they were derived. Of course, many of
the amino acids in the framework region make little or no
35   direct contribution to the specificity or affinity of an
antibody. Thus, many individual conservative substitutions of
framework residues can be tolerated without appreciable change
of the specificity or affinity of the resulting humanized

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immunoglobulin. However, in general, such substitutions are undesirable.

(5) Production of Variable Regions

Having conceptually selected the CDR and framework components of humanized immunoglobulins, a variety of methods are available for producing such immunoglobulins. Because of the degeneracy of the code, a variety of nucleic acid sequences will encode each immunoglobulin amino acid sequence. The desired nucleic acid sequences can be produced by *de novo* solid-phase DNA synthesis or by PCR mutagenesis of an earlier prepared variant of the desired polynucleotide. Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion and insertion variants of target polypeptide DNA. *See* Adelman et al., *DNA* 2:183 (1983). Briefly, the target polypeptide DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a single-stranded DNA template. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that incorporates the oligonucleotide primer, and encodes the selected alteration in the target polypeptide DNA.

(6) Selection of Constant Region

The variable segments of humanized antibodies produced as described *supra* are typically linked to at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Human constant region DNA sequences can be isolated in accordance with well-known procedures from a variety of human cells, but preferably immortalized B-cells (*see* Kabat et al., *supra,* and WO87/02671) (each of which is incorporated by reference in its entirety for all purposes). Ordinarily, the antibody will contain both light chain and heavy chain constant regions. The heavy chain constant region usually includes CH1, hinge, CH2, CH3, and CH4 regions.

The humanized antibodies include antibodies having all types of constant regions, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4.

When it is desired that the humanized antibody exhibit
cytotoxic activity, the constant domain is usually a
complement-fixing constant domain and the class is typically
IgG_1. When such cytotoxic activity is not desirable, the
constant domain may be of the IgG_4 class. The humanized
antibody may comprise sequences from more than one class or
isotype.

(7)   Expression Systems

Nucleic acids encoding humanized light and heavy
chain variable regions, optionally linked to constant regions,
are inserted into expression vectors. The light and heavy
chains can be cloned in the same or different expression
vectors. The DNA segments encoding immunoglobulin chains are
operably linked to control sequences in the expression
vector(s) that ensure the expression of immunoglobulin
polypeptides. Such control sequences include a signal
sequence, a promoter, an enhancer, and a transcription
termination sequence. Expression vectors are typically
replicable in the host organisms either as episomes or as an
integral part of the host chromosomal DNA. Commonly,
expression vectors will contain selection markers, e.g.,
tetracycline or neomycin, to permit detection of those cells
transformed with the desired DNA sequences (see, e.g., U.S.
Patent 4,704,362).

E. coli is one prokaryotic host useful particularly
for cloning the polynucleotides of the present invention.
Other microbial hosts suitable for use include bacilli, such
as Bacillus subtilus, and other enterobacteriaceae, such as
Salmonella, Serratia, and various Pseudomonas species. In
these prokaryotic hosts, one can also make expression vectors,
which will typically contain expression control sequences
compatible with the host cell (e.g., an origin of
replication). In addition, any number of a variety of well-
known promoters will be present, such as the lactose promoter
system, a tryptophan (trp) promoter system, a beta-lactamase
promoter system, or a promoter system from phage lambda. The
promoters will typically control expression, optionally with

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an operator sequence, and have ribosome binding site sequences
and the like, for initiating and completing transcription and
translation.

     Other microbes, such as yeast, may also be used for

5   expression. *Saccharomyces* is a preferred host, with suitable
vectors having expression control sequences, such as
promoters, including 3-phosphoglycerate kinase or other
glycolytic enzymes, and an origin of replication, termination
sequences and the like as desired.

10   In addition to microorganisms, mammalian tissue cell
culture may also be used to express and produce the
polypeptides of the present invention (*see* Winnacker, *From
Genes to Clones* (VCH Publishers, N.Y., N.Y., 1987).
Eukaryotic cells are actually preferred, because a number of

15   suitable host cell lines capable of secreting intact
immunoglobulins have been developed. Preferred suitable host
cells for expressing nucleic acids encoding the
immunoglobulins of the invention include: monkey kidney CV1
line transformed by SV40 (COS-7, ATCC CRL 1651); human

20   embryonic kidney line (293) (Graham et al., *J. Gen. Virol.*
36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10);
Chinese hamster ovary-cells-DHFR (CHO, Urlaub and Chasin,
Proc. Natl. Acad. Sci. (USA) 77:4216 (1980)); mouse sertoli
cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey

25   kidney cells (CV1 ATCC CCL 70); african green monkey kidney
cells (VERO-76, ATCC CRL 1587); human cervical carcinoma cells
(HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34);
buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung
cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB

30   8065); mouse mammary tumor (MMT 060562, ATCC CCL51); and, TRI
cells (Mather, et al., *Annals N.Y. Acad. Sci.* 383:44-46
(1982)); baculovirus cells.

     The vectors containing the polynucleotide sequences
of interest (*e.g.*, the heavy and light chain encoding

35   sequences and expression control sequences) can be transferred
into the host cell by well-known methods, which vary depending
on the type of cellular host. For example, calcium chloride
transfection is commonly utilized for prokaryotic cells.

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whereas calcium phosphate treatment or electroporation may be
used for other cellular hosts. (*See generally* Sambrook et
al., *Molecular Cloning: A Laboratory Manual* (Cold Spring
Harbor Press, 2nd ed., 1989) (incorporated by reference in its
entirety for all purposes). When heavy and light chains are
cloned on separate expression vectors, the vectors are co-
transfected to obtain expression and assembly of intact
immunoglobulins. After introduction of recombinant DNA, cell
lines expressing immunoglobulin products are cell selected.
Cell lines capable of stable expression are preferred (i.e.,
undiminished levels of expression after fifty passages of the
cell line).

Once expressed, the whole antibodies, their dimers,
individual light and heavy chains, or other immunoglobulin
forms of the present invention can be purified according to
standard procedures of the art, including ammonium sulfate
precipitation, affinity columns, column chromatography, gel
electrophoresis and the like (*see generally* Scopes, *Protein
Purification* (Springer-Verlag, N.Y., 1982). Substantially
pure immunoglobulins of at least about 90 to 95% homogeneity
are preferred, and 98 to 99% or more homogeneity most
preferred, for pharmaceutical uses.

The recombinant techniques described above can also
be used for expression of native sequences encoding human or
murine antibodies. This approach is particularly advantageous
for expression of human antibodies that are isolated as
unstable cell lines.

III. Antibodies Fragments

In another embodiment of the invention, fragments of
the intact antibodies described above are provided.
Typically, these fragments compete with the intact antibody
from which they were derived for specific binding to P-
selectin, and bind with an affinity of at least 10^7, 10^8, 10^9
M^{-1}, or 10^{10} M^{-1}. Antibody fragments include separate heavy
chains, light chains Fab, Fab' F(ab')$_2$, Fabc, and Fv.
Fragments can be produced by enzymic or chemical separation of
intact immunoglobulins. For example, a F(ab')$_2$ fragment can

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be obtained from an IgG molecule by proteolytic digestion with
pepsin at pH 3.0-3.5 using standard methods such as those
described in Harlow and Lane, Antibodies: A Laboratory Manual,
Cold Spring Harbor Pubs., N.Y. (1988). Fab fragments may be
5   obtained from F(ab')$_2$ fragments by limited reduction, or from
whole antibody by digestion with papain in the presence of
reducing agents. (See id.) Fragments can also be produced by
recombinant DNA techniques. Segments of nucleic acids
encoding selected fragments are produced by digestion of full-
10   length coding sequences with restriction enzymes, or by de
novo synthesis. Often fragments are expressed in the form of
phage-coat fusion proteins. This manner of expression is
advantageous for affinity-sharpening of antibodies as
discussed in Section IV.

15

IV.   Affinity-Sharpened Antibodies
          Many of the immunoglobulins described above can
undergo non-critical amino-acid substitutions, additions or
deletions in both the variable and constant regions without
20   loss of binding specificity or effector functions, or
intolerable reduction of binding affinity (i.e., below about
10^7 M^{-1}). Usually, immunoglobulins incorporating such
alterations exhibit substantial sequence identity to a
reference immunoglobulin from which they were derived.
25   Occasionally, a mutated immunoglobulin can be selected having
the same specificity and increased affinity compared with a
reference immunoglobulin from which it was derived. Phage-
display technology offers powerful techniques for selecting
such immunoglobulins. See, e.g., Dower et al., WO 91/17271
30   McCafferty et al., WO 92/01047; and Huse, WO 92/06204.

V.   Hybrid Antibodies
          The invention also provides hybrid antibodies that
share the specificity of blocking antibodies against P-
35   selectin but are also capable of specific binding to a second
moiety. In hybrid antibodies, one heavy and light chain pair
is usually from an anti-P-selectin antibody and the other pair
from an antibody raised against another epitope. This results

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in the property of multi-functional valency, i.e., ability to
bind at least two different epitopes simultaneously, where at
least one epitope is the epitope to which the blocking P-
selectin antibody binds. Such hybrids can be formed by fusion
5   of hybridomas producing the respective component antibodies,
or by recombinant techniques.

Immunoglobulins can also be fused to functional
regions from other genes (e.g., enzymes) to produce fusion
proteins (e.g., immunotoxins) having novel properties.

10

VI. Nucleic Acids

The intact antibodies and antibody fragments
described above are often produced by expression of nucleic
acids. All nucleic acids encoding any antibody or antibody
15   described in this application are expressly included in the
invention. Modifications of nucleic acids are readily
accomplished by a variety of well-known techniques, such as
site-directed mutagenesis (*see* Gillman and Smith, *Gene*, 8:81-
97 (1979) and Roberts, et al., *Nature*, 328:731-734 (1987)).
20   Many of the nucleic acids of the invention show substantial
sequence identity to nucleic acids encoding the heavy and
light chains of mu MAb PB1.3 or the exemplified humanized
derivatives thereof.

25   ## VII. Computers

In another aspect of the invention, computers
programmed to display three dimensional images of antibodies
on a monitor or printer are provided. For example, a Silicon
Graphics IRIS 4D workstation running under the UNIX operating
30   system and using the molecular modelling package QUANTA
(Polygen Corp. USA) is suitable. Computers are useful for
visualizing models of variants of humanized antibodies. In
general, the antibodies of the invention already provide
satisfactory binding affinity. However, it is likely that
35   antibodies with even stronger binding affinity could be
identified by further variation of certain amino acid
residues. The three dimensional image will also identify many
noncritical amino acids, which could be the subject of

conservative substitutions without appreciable affecting the
binding affinity of the antibody.

VIII.   Large-Scale Production of Antibodies

5   For large-scale production of antibodies, stable
cells are produced containing multiple copies of the genes
encoding heavy and light chain immunoglobulins in their
chromosomes. Multiple copies are obtained by amplification of
isolated region(s) of a cell's chromosomal DNA. Amplification
10   is achieved using a selection agent, e.g., methotrexate (MTX),
that inactivates a cellular enzyme (e.g., DHFR) that is
essential under certain growth conditions. Amplification,
i.e., the accumulation of multiple copies of the DHFR gene,
results in greater amounts of DHFR being produced in response
15   to greater amounts of MTX. Amplification pressure is applied
notwithstanding the presence of endogenous DHFR, by adding
ever greater amounts of MTX to the media. Amplification of
desired genes (here genes encoding immunoglobulin heavy and
light chains) is achieved by linking each gene to a copy of a
20   DHFR gene on the same or separate plasmid(s), and
cotransfecting the plasmid(s). Amplification of the DHFR
genes in response to MTX results in a concomitant increase in
copy number of the desired immunoglobulin genes. Increased
copy number of the desired genes results in greater expression
25   of the desired heterologous protein. Stable cell lines
expressing about 10-100, 20-50 or about 30 μg humanized
immunoglobulin per 10^6 cells per day are preferred.

IX.   P-Selectin Fragments

30   In another aspect of the invention, fragments of P-
selectin are provided. The fragments comprise amino acids
from the fifth C3b-C4b regulatory domain of P-selectin (fifth
CRP). The fragments contain the epitope bound by mu MAb PB
1.3 and/or proximal epitopes bound by competing antibodies.
35   The fragments usually contain up to 5, 10, 20, 25, 50, 75, 100
or 200 amino acids in total. Most fragments contain some or
all of the amino acids between positions 448 and 467 of the
human P-selectin sequence shown in Fig. 34. These amino acids

define the epitope specifically bound by mu MAb PB1.3. In
many fragments, at least five contiguous amino are from
between positions 448 and 467 of the amino acid sequence shown
in Fig. 34. Other fragments contain the entire contiguous
5   segment of amino acids between positions 448 and 467. Some
fragments consist essentially of this contiguous segment of
amino acids. In these fragments, no other amino acids are
present that contribute to the binding specificity or affinity
of the fragment. Often, no other amino acids of any
10   description are present.

The polypeptide fragments of the invention have a
variety of uses. The fragments are useful as immunogens for
generating antibodies that compete with mu MAb PB 1.3 for
specific binding to P-selectin. The use of defined fragments
15   as immunogens reduces the extent of screening required to
isolate an antibody of desired specificity. The polypeptide
fragments are also useful in the diagnostic and therapeutic
methods described *infra*. Some fragments compete with full-
length P-selectin molecules for binding to activated
20   neutrophils. Thus, these fragments are useful for aborting
diseases and conditions of the immune system mediate by
interactions of P-selectin with its ligand(s) on activated
neutrophils. Some fragments are also useful for monitoring
the presence of activated neutrophils by binding to ligands to
25   P-selectin present on the surface of these cells. The small
size of the fragments renders them particularly suitable for
effective delivery following *in vivo* administration. The
exclusively human origin of the fragments reduces the risk of
side-effects that might occur with some other agents.
30   X.   Therapeutic and Diagnostic Methods

The therapeutic methods employ the antibodies (whole
and binding fragments), and fragments of P-selectin discussed
above as therapeutic agents for treatment of various diseases.
The therapeutic agents are useful for prophylactic and
35   therapeutic treatment of a wide variety of diseases and
disorders of the immune system. Such diseases and disorders
include transplant rejection, graft versus host disease,
autoimmune diseases such as insulin-dependent diabetes

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mellitus, multiple sclerosis, stiff man syndrome, rheumatoid
arthritis, myasthenia gravis and lupus erythematosus, and
inflammatory disorders. The agents are also useful for
preventing tumor metastasis inhibiting the adhesion of
5   circulating cancer cells, such as carcinomas of the colon and
melanoma. Some therapeutic agents function by blocking or
otherwise antagonizing the action of a P-selectin molecule
with its ligand. Other therapeutic agents function by killing
cells bearing a polypeptide against which the agent is
10   targeted.

The therapeutic agents are particularly suitable for
treatment of inflammatory and thrombotic conditions including
post-ischemic leukocyte-mediated tissue damage (reperfusion
injury) arising from traumatic shock, stroke, myocardial
15   infarction, acute transplantation rejection, frost-bite
injury, compartment syndrome, and pathophysiologic conditions
associated with cardio-pulmonary bypass, acute leukocyte-
mediated lung injury (e.g., adult respiratory distress
syndrome), septic shock, wound associated sepsis secondary to
20   viral infection by e.g., herpes simplex virus, IgE-mediated
allergic reactions such as acute phase asthmatic disease, and
chronic inflammatory conditions, including rheumatoid
arthritis, atopic dermatitis and psoriasis.

Ischemia/reperfusion injury is an inflammatory
25   condition that occurs on restoring blood flow to organs
suffering from an obstructed supply causing ischemia (oxygen
deprivation). Unless rapidly relieved by reperfusion,
ischemia causes death of surrounding cells, and eventually,
death of a whole organ or patient. However, accumulating
30   evidence suggests that reperfusion may itself exert
deleterious effects on surrounding tissue. The deleterious
effects of reperfusion are believed to result at least in part
from an inflammatory response mediated by activated
neutrophils in the restored blood flow. Some patients have
35   whole-body ischemia, whereas in other patients ischemia is
confined to particular parts or organs of the body. For
example, a patient may suffer from epidermal, myocardial,
renal, cerebral, splenic, hepatic, spinal, splanchnic,

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pulmonary, partial-body, or whole-body ischemia. The
therapeutic agents of the invention function by antagonizing
the interaction of such lymphocytes with P-selectin.

One major component of acute allergic conditions,
including asthma, is the degranulation of mast cells following
challenge of subjects with antigens to which they are
specifically sensitized. The consequences of mast cell
degranulation include a bronchoconstrictor response and also
an inflammatory response characterized in part by leukocyte
accumulation. Histamine, which is contained together with
other inflammatory mediators in mast cells, can induce the
expression of P-selectin on vascular endothelial cells,
indicating that degranulation of mast cells may also result in
P-selectin expression and subsequent leukocyte accumulation.
Because mast cell degranulation is a key element in the
pathogenesis of allergic conditions, administration of
antibodies to P-selectin will be useful for the treatment of
human allergic conditions.

The methods are particularly useful for humans, but
may also be practiced on veterinary subjects. The therapeutic
methods are usually applied to organs present in living
subjects. However, some methods, such as ischemia-reperfusion
therapy, are equally applicable to dissected organs,
particularly when such organs are awaiting transplant to a
recipient patient. Therapeutic methods can also be performed
ex vivo.

Therapeutic agents and compositions targeted against
P-selectin can also be used in combination with agents
targeted against other molecules, particularly humanized or
human antibodies reactive with different adhesion molecules.
Suitable immunoglobulins include those specific for CD11a,
CD11b, CD18, E-selectin, L-selectin and ICAM-1. The
immunoglobulins should bind to epitopes of these adhesion
molecules so as to inhibit binding of leukocytes, particularly
neutrophils, to endothelial cells. Other suitable antibodies
for use in combination therapies are those specific for
lymphokines, such as IL-1, IL-2 and IFN-γ, and their
receptors. Such antibodies serve to block activation of

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endothelial cells, and thereby prevent their interaction with
neutrophils in an inflammatory response.

    The blocking P-selectin antibodies and pharma-
ceutical compositions of this invention are particularly
5   useful for parenteral administration, i.e., subcutaneously,
intramuscularly or intravenously. A number of new drug
delivery approaches are being developed, the pharmaceutical
compositions of the present invention are suitable for
administration using these new methods, as well. *See* Langer,
10 *Science*, 249:1527-1533 (1990).

    The blocking P-selectin antibodies can be directly
or indirectly coupled to the chemotherapeutic agent. The
coupling, which may be performed by means, generally known in
the art, should not substantially inhibit the ability of the
15 immunoglobulin to bind the receptor nor should it
substantially reduce the activity of the chemotherapeutic
agent. A variety of chemotherapeutics can be coupled for
targeting. For example, anti-inflammatory agents which may be
coupled include immunomodulators, platelet activating factor
20 (PAF) antagonists, cyclooxygenase inhibitors, lipoxygenase
inhibitors, and leukotriene antagonists. Some preferred
moieties include cyclosporin A, indomethacin, naproxen, FK-
506, mycophenolic acid, and the like. Similarly, anti-
oxidants, e.g., superoxide dismutase, are useful in treating
25 reperfusion injury. Likewise, anticancer agents, such as
daunomycin, doxorubicin, vinblastine, bleomycin, and the like
can be targeted.

    The P-selectin targeting may also be accomplished
via amphipaths, or dual character molecules (polar:nonpolar)
30 which exist as aggregates in aqueous solution. Amphipaths
include nonpolar lipids, polar lipids, mono- and diglycerides,
sulfatides, lysolecithin, phospholipids, saponin, bile acids
and salts. These molecules can exist as emulsions and foams,
micelles, insoluble monolayers, liquid crystals, phospholipid
35 dispersions and lamellar layers. These are generically
referred to herein as liposomes. In these preparations, the
drug to be delivered is incorporated as part of a liposome in
which an anti-P-selectin immunoglobulin is embedded. In this

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embodiment, the immunoglobulin need not bind a functional epitope on the P-selectin molecule, so long as the immunoglobulin effectively targets the liposome to P-selectin molecules. When the liposomes are brought into proximity of

5   the affected cells, they deliver the selected therapeutic compositions.

A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al., *Ann. Rev. Biophys. Bioeng.* 9:467 (1980), U.S. Pat. Nos. 4,235,871,

10   4,501,728 and 4,837,028, incorporated herein by reference. Targeting of liposomes using a variety of targeting agents (e.g., ligands, receptors and monoclonal antibodies) is well known in the art. (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044, both of which are incorporated herein by

15   reference). Standard methods for coupling targeting agents to liposomes can be used. Antibody targeted liposomes can be constructed using, for instance, liposomes which incorporate protein A (*see* Renneisen, et al., *J. Biol. Chem.*, 265:16337– 16342 (1990) and Leonetti et al., *Proc. Natl. Acad. Sci. (USA)*

20   87:2448–2451 (1990).

Pharmaceutical compositions for parenteral administration usually comprise a solution of a therapeutic agent (e.g., an antibody against P-selectin (intact or binding fragment or a P-selectin fragment) or a cocktail of several

25   such agents dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by

30   conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH-adjusting and buffering agents, tonicity adjusting agents and the like, for example sodium acetate, sodium

35   chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of antibody in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 0.1% to as much as 1.5% or 2.0%

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by weight and will be selected primarily based on fluid
volumes, viscosities, etc., in accordance with the particular
mode of administration selected. Actual methods for preparing
parenterally administrable compositions will be known or
apparent to those skilled in the art and are described in more
detail in, for example, Remington's Pharmaceutical Sciences,
17th Ed., Mack Publishing Company, Easton, Pennsylvania
(1985), which is incorporated herein by reference.

        The antibodies of this invention can be lyophilized
for storage and reconstituted in a suitable carrier prior to
use. This technique has been shown to be effective with
conventional immunoglobulins and art-known lyophilization and
reconstitution techniques can be employed. Lyophilization and
reconstitution can lead to varying degrees of antibody ac-
tivity loss (e.g., with conventional immunoglobulins, IgM
antibodies tend to have greater activity loss than IgG
antibodies) and that use levels may have to be adjusted to
compensate.

        The compositions containing the present antibodies
or a cocktail thereof can be administered for the prophylactic
and/or therapeutic treatments. In therapeutic application,
compositions are administered to a patient in an amount
sufficient to cure or at least partially arrest the infection
and its complications. An amount adequate to accomplish this
is defined as a "therapeutically effective dose." Amounts
effective for this use will depend upon the severity of the
disease and the general state of the patient's own immune
system, but generally range from about .05 mg/kg body weight
to about 5 mg/kg body weight, preferably between about .2
mg/kg body weight to about 1.5 mg/kg body weight.

        The materials of this invention may generally be
employed in serious disease states, that is, life-threatening
or potentially life-threatening situations. In such cases, in
view of the minimization of extraneous substances and the
lower probability of "foreign substance" rejections (e.g.,
HAMA) which are achieved by human or humanized antibody forms
of this invention, it is possible and may be felt desirable by

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the treating physician to administer substantial excesses of these antibodies.

In prophylactic applications, compositions containing the present antibodies or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity, but is generally in the ranges described above.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the immunoglobulins of this invention sufficient to treat the patient effectively.

The antibodies of invention (whole and binding fragments) and fragments of P-selectin can also be used for diagnostic purposes. An amount sufficient for these purposes is defined to be a "diagnostically effective dose." In diagnostic uses, the precise amounts will depend upon the patient's state of health, mode of administration, and the like. The antibodies may either be labeled or unlabeled. Unlabeled antibodies can be used in combination with other labeled antibodies (second antibodies) that are reactive with the antibody, such as antibodies specific for the particular immunoglobulin constant region. Alternatively, the antibodies can be directly labeled. A wide variety of labels may be employed, such as radionuclides, fluorescers, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, ligands (particularly haptens).

The antibodies (whole and binding fragments) are useful for detecting the presence of cells bearing the P-selectin receptor. The presence of such cells is diagnostic of an inflammatory condition or disease and may signal the need for commencement of a therapeutic method discussed *supra*. Diagnosis can be accomplished by removing a cellular sample from a patient. The amount of expressed P-selectin receptor

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in individual cells of the sample is then determined, *e.g.*, by immunohistochemical staining of fixed cells or by Western blotting of a cell extract with an antibody of the invention. Fragments of P-selectin are useful for monitoring the presence

5   of activated neutrophils, a presence that can be indicate of an undesirable immune response.

Diagnosis can also be achieved by *in vivo* administration of a labelled antibody (preferably a humanized or human antibody) and detection by *in vivo* imaging. The

10   concentration of MAb administered should be sufficient that the binding to cells having the target antigen is detectable compared to the background signal. The diagnostic reagent can be labelled with a radioisotope for camera imaging, or a paramagnetic isotope for magnetic resonance or electron spin

15   resonance imaging.

A change (typically an increase) in the level of P-selectin protein in a cellular sample or imaged from an individual, which is outside the range of clinically established normal levels, may indicate the presence of an

20   undesirable inflammatory response reaction in the individual from whom the sample was obtained, and/or indicate a predisposition of the individual for developing (or progressing through) such a reaction. P-selectin can also be employed as a differentiation marker to identify and type

25   cells of certain lineages and developmental origins. Such cell-type specific detection can be used for histopathological diagnosis of undesired immune responses.

Kits can also be supplied for use with the subject antibodies. Thus, the subject antibody composition of the

30   present invention may be provided, usually in a lyophilized form in a container, either alone or in conjunction with additional antibodies specific for the desired cell type. The antibodies, which may be conjugated to a label or toxin, or unconjugated, are included in the kits with buffers, such as

35   Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., serum albumin, or the like, and a set of instructions for use. Generally, these materials will be present in less than about 5% wt. based on the amount of

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active antibody, and usually present in total amount of at
least about 0.001% wt. based again on the antibody
concentration. Frequently, it will be desirable to include an
inert extender or excipient to dilute the active ingredients,
5   where the excipient may be present in from about 1 to 99% wt.
of the total composition. Where a second antibody capable of
binding to an anti-P-selectin antibody is employed in an
assay, the second antibody will usually be present in a
separate vial. The second antibody is typically conjugated to
10   a label and formulated as described above.

The following examples are offered by way of
illustration, not by limitation.

Example 1
Preparation of Reagents

15   This example describes preparation or isolation of
reagents used for the immunizations in Examples 2 and 3 and
the ELISAs in Example 4.

20   A.   "OUTDATED PLATELETS"

A preparation of platelets which was purified from
"outdated platelets" obtained from the San Diego Blood Bank.
The method used was a modification of the method described in
Moore et al., *J. Cell. Biol.* 112:491-499 (1991), which is
25   incorporated herein by reference. Briefly, 25 units of
outdated platelet-rich plasma were centrifuged twice for 10
min at 1200rpm (300xg) to remove contaminating non-platelet
blood cells. The purified platelet preparation was then
washed three times with a buffer containing 0.1M NaCl, 20mM
30   Tris and 5mM Benzamidine. It also contained 3.8% sodium
citrate. The pH was adjusted to 7.5 with 1N HCl. The
purified platelets were stored at 70°C and were washed once in
PBS before injection.

35   B.   PURIFIED P-SELECTIN

Fractionation of Platelets: Washed outdated
platelets were fractionated essentially according to Moore et
al., 1991, *supra*, 25 units washed platelets were made 100 uM

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in leupeptin and 1 mM in 4-(2aminoethyl)-benzenesulfonyl-
fluoride (AEBSF), freeze-thawed three times in dry
ice/methanol, and homogenized ten strokes with a Dounce
homogenizer. The suspension was then centrifuged in a Beckman
5   Ti 70 rotor at 4°C at 35,000 rpm for 60 min. The supernatant
from this spin was the starting material for the purification
of "soluble" P-selectin. The pellet was resuspended in 30 ml
1 mM $MnCl_2$, 1 mM $CaCl_2$, 5 mM benzamidine-HCl, 0,1M NaCl, 20 mM
Tris, 2% Triton X-100, pH 7.5, homogenized 10 strokes with a
10   Dounce homogenizer, and incubated for 1 h at 4°C. The
suspension was then centrifuged for 1 h in a Beckman Ti 70
rotor at 4°C at 35,000 rpm. The supernatant from this spin
was the starting material for the isolation of
"membrane-bound" P-selectin.

15   <u>Isolation of P-Selectin from Fractionated Platelets</u>:
"Soluble" and "membrane-bound" P-selectin were then further
purified separately by some or all of the following
procedures. The non-ionic detergent Rennex 30 (Accurate
Chemical and Scientific Corp.) was included at a concentration
20   of 0.1% in all buffers for isolation of the membrane-bound
form of P-selectin. All buffers also included 1 mM $CaCl_2$ and
1 mM $MnCl_2$. Fractions containing P-selectin were detected by
Western blotting with the monoclonal antibody PNB1.6.

            <u>Lentil Lectin Chromatography</u>: "Soluble P-selectin"
25   supernatant or "membrane-bound P-selectin" detergent extract
from 25 units of platelets were passed over a column (1.5 x
5.5 cm) of lectin from Lens culinaris (lentil lectin) coupled
to Sepharose 4B (Sigma Chemical Co., L-0511). The column was
then washed with 150 ml 20 mM Tris, 0.1M NaCl, pH 7.5. Bound
30   glycoproteins were then eluted from the column with 10 x 3 ml
aliquots of 20 mM Tris, 0.5M NaCl, 0.5M alpha-methyl D-
mannopyranoside, pH 7.5. Fractions containing P-selectin were
concentrated to 1.5-2 ml in an Amicon Centriprep 30.

            <u>Gel Filtration Chromatography</u>: Concentrated
35   fractions from lentil lectin chromatography were injected onto
a Toso Haas G3000 SW HPLC column (21.5 mm x 30 cm)
equilibrated with 20 mM Tris, 0.1M NaCl, pH 7.5. The column
was eluted with the same buffer at a flow rate of 0.75 ml/min.

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fractions (1.5 ml) were collected, and analyzed by Western blotting. Fractions containing P-selectin were pooled and concentrated to 1.5-2 ml in an Amicon Centriprep 30, followed by several buffer exchanges with 20 mM Tris, pH 7.5.

5   Heparin-Agarose Chromatography: The sample, in 20 mM Tris, pH 7.5, was applied to a 1 ml column of Heparin-Agarose, the column was washed with the same buffer, and eluted with 20 mM Tris, 1.5M NaCl, pH 7.5.

          Anion Exchange Chromatography: Fractions were
10   dialyzed into 20 mM Tris, pH 7.5, and injected onto a Toso Haas DEAE-5PW column (7.5 mm x 7.5 cm) that had been equilibrated with 20 mM Tris, pH 7.5, and eluted with a salt gradient to 1M NaCl. Fractions containing P-selectin were pooled, and concentrated in an Amicon Centriprep-30, and
15   stored at -80°C.

          Cation Exchange Chromatography: Samples were dialyzed into 10 mM phosphate, pH 7, and injected onto a Poros CM/P (4.6/100) column that had been equilibrated in the same buffer, and eluted with a salt gradient to 1M NaCl.

20

C.   ISOLATION AND ACTIVATION OF FRESH HUMAN PLATELETS
          All chemicals used throughout the platelet isolation procedure were obtained from Sigma Chemical Company, St. Louis, MO.

25   1.   42 ml of blood was drawn from a human volunteer
              blood donor into a syringe containing 7 ml Acid
              Citrate Dextrose anticoagulant (ACD).
              Preparation of ACD anticoagulant
              Dextrose   2.0g
30   Sodium Citrate   2.49g
              Citric Acid   1.25g
              Bring to 100 ml with distilled water
        2.   The needle was removed and the blood was transferred
              to two sterile 50 ml tubes.
35   3.   The tubes were centrifuged in an IECC-6000
              centrifuge equipped with a 921 head (radius=17.2
              cm.) at 800 rpm (approx. 90xg) for 15 min at room
              temperature with the brake off.

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4. The supernatant was removed using a plastic pipette. The supernatant was removed as close as possible to the buffy layer.

5. The supernatant was centrifuged at 1200 rpm (approx. 300xg) for 6 min.

6. The supernatant was removed.

7. The supernatant was centrifuged at 2000 rpm (approx. 1200xg) for 10 min. The platelets appeared as a cell button at the bottom of the tube. The supernatant was discarded.

8. The platelets were washed as follows: 2 ml Tyrode-Hepes Buffer pH 6.5, containing Prostaglandin, E_1 (PGE_1 at a final concentration of 100nM was added and the platelets were gently resuspended. A further 10 ml of the same buffer was added and the sample was centrifuged at 3000 rpm for 10 min.

Preparation of Tyrode-Hepes Buffer

NaCl	8.0g
KCl	0.2g
$NaH_2PO_4H_2O$	0.057g
$MgCl_26H_2O$	0.184g
$NaHCO_3$	0.1g
Dextrose	1.0g
HEPES	2.383g

Bring to 1 liter with distilled water. Adjust pH to 6.5 with 1N NaOH.

9. Step #8 was repeated once more and then the platelets were washed once in the same buffer without PGE_1.

10. The platelets were counted in a Coulter Counter and diluted to 2×10^8/ml.

11. The pH of the platelet suspension was adjusted to 7.2, and the amount of thrombin (Human thrombin-Sigma) required for maximal activation was determined. There is a certain amount of donor variation, but optimal activation is normally obtained in the range of 0.25 - 0.5 Units/ml. Maximal activation i.e., maximal thrombin-induced

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aggregation appears to correspond to maximal
expression of P-selectin.

12. The required amount of thrombin was added to the
platelet preparation and the mixture was allowed to
stand at room temperature for 20 min without
stirring to prevent aggregation.

Example 2
Preparation of Blocking P-selectin Antibody

This example describes preparation of mu mAB PB1.3,
a monoclonal antibody to P-selectin that inhibits binding of
thrombin-activated platelets to neutrophils.

One RBF/DnJ male mouse, received from Jackson
Laboratories, was used as a source of antibody producing cells
and was immunized according to the following schedule (all
injections were performed intraperitoneally):

1. Month 1 - 200 μl of packed "outdated platelets"
containing approximately 6×10^9 platelets

2. Month 2 - 250 μl of packed "outdated platelets"
(8×10^9 platelets)

3. Month 4 - 250 μl of packed "outdated platelets"
(8×10^9 platelets)

4. Month 5 - "Soluble" fraction of P-selectin,
isolated on lentil lectin.

5. Month 6 - "Soluble" P-selectin, purified by
lentil lectin, gel filtration, heparin-agarose,
and ion-exchange chromatography.

Details of the preparation of all agents used for
immunization are presented in Example 1.

A. MYELOMA FUSION CELL LINE

FOX-NY, a mouse myeloma cell line deficient for
adenosine phosphoribosyltransferase (APRT) and hypoxanthine
phosphoribosyltransferase (HPRT) was obtained from the
American Type Culture Collection (CRL 1732) and maintained in
RPMI 1640 containing 10% fetal bovine serum (Hyclone) and 1%
L-glutamine.

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B.   CELL FUSION PROCEDURE

Four days after the final boost the spleen was removed and 1.2×10^8 splenocytes were recovered. These were fused with FOX-NY myeloma cells using PEG 1500 (BMB) using the following protocol: The isolated splenocytes were washed twice in serum-free cell culture medium. Splenocytes and myeloma cells were combined at a ratio of 1:4.8 (myelomas to spleen cells). The combined cell pellet was washed twice in serum free medium, then aspirated to dryness. The cell pellet was resuspended by gentle tapping and heated in a waterbath at 37°C for 1 min. The pellet was distributed around the sides and bottom of a 50 ml conical centrifuge tube. One ml of PEG 1500 (50% w/v in 75mM HEPES, BMB Lot #14702800) previously warmed to 37°C was added over 60 seconds while rotating the tube to maintain a thin layer of cells. One ml of serum free medium was added slowly over 60 seconds. An additional 1ml of medium was added slightly faster. A further 8 ml of medium was added and the tube was allowed to stand undisturbed for 8 min and was then centrifuged for 5 min at 300xg. The final pellet was resuspended in RPMI 1640 containing 10% fetal bovine serum Hyclone), 1% L-glutamine, 1% Sodium Pyruvate, and 1xAAT (Sigma). The cells were plated in 10 flat bottom 96 well microtiter plates (COSTAR). No feeder cells were used.

Example 3
Preparation of Non-blocking Anti-P-selectin antibody

This example describes preparation of mAB PNB1.6 a monoclonal antibody to P-selectin that does not inhibit binding of thrombin-activated platelets to neutrophils.

One RBF/DnJ male mouse, received from Jackson Laboratories, was used as a source of antibody producing cells and was immunized according to the following schedule (all injections were performed intraperitoneally):

1.   Month 1 - 100μl of thrombin-activated freshly isolated platelets (approximately 3×10^9 platelets)

2.   Month 2 - 200μl of thrombin-activated fresh platelets (6×10^9 platelets)

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3.   Month 4 - 100μl of thrombin-activated fresh
      platelets (3x10^9 platelets)

Four days post final boost, the spleen was removed
and the splenocytes were recovered. They were fused with FOX-
NY myeloma cells using PEG 1500(Sigma) generally as described
in Oi et al., "Immunoglobulin-Producing Hybrid Cell Lines" in
Selected Methods in Cellular Immunology, eds. Mishell and
Shiigi, pp 351-372, 1980, which is incorporated herein by
reference.

Example 4
Screening for Antibody

This example describes screening of supernatant
media from fused cells produced in Examples 2 and 3.
Supernatants from Example 2 were tested by ELISA assay against
(a) thrombin-activated platelets, (b) purified P-selectin and
(c) recombinant P-selectin. Supernatants from Example 2 were
tested by ELISA assay against thrombin-activated platelets.
Each of these assays is described below. Preparation of the
reagents used in each of the assays is described in Example 1.

A.   THROMBIN-ACTIVATED PLATELETS
      1.   A 96 well flat bottom COSTAR plate was coated with
            0.1% gelatin (2% gelatin-Sigma) by adding 100μl/well
            and incubating at 37°C for 15 min.
      2.   The gelatin was removed and 100 μl of thrombin
            activated platelets (10^8/ml) were added to each
            well.
      3.   The plate was incubated at 37°C for 15 min.
      4.   The plate was centrifuged at 800 rpm (90xg) for 2
            min.
      5.   The unbound platelets were removed and the plate was
            washed x2 with PBS.
      6.   100μl of PBS containing 1% BSA were added to each
            well and the plate was allowed to stand at room
            temperature for 60 min.

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7.   100µl of supernatant were added to each well. The plate was placed on a shaker at room temperature for 60 min.

8.   The plate was washed x4 with PBS.

5   9.   100µl of peroxidase-conjugated goat anti-mouse IgG diluted 1/1000 in PBS containing 1% BSA was added to each well. The plate was allowed to stand at room temperature for 30 min.

10. The plate was washed x7 with PBS.

10   11. 100µl of ABTS substrate system (Kirkegaard and Perry, Laboratories, Inc.) was added to each well.

12. The plate was allowed to develop at room temperature for up to 30 min.

13. The OD was measured at 414nm in a Titertek Multiskan

15   MCC/340.

B.   PURIFIED P-selectin

1.   "Soluble" or "membrane-bound" P-selectin, isolated by chromatography on lentil lectin, gel filtration,

20   and DEAE columns, was diluted (1:50 to 1:1000) in DPBS (Dulbecco's Phosphate Buffered Saline is Phosphate-buffered saline (PBS) containing $CaCl_2$(1mM) and $MgSO_4$ (0.5mM)) and coated overnight at 4°C onto Falcon 96 well microtiter plates.

25   2.   The plates were then blocked for 1 h or longer with DPBS+1%BSA.

3.   Supernatants to be assayed were then added to the wells, and incubated for 30-60 min at room temp.

4.   The plates were washed with DPBS, and then sheep

30   anti-mouse IgG horseradish peroxidase conjugate (Sigma A-6782, 1:1000 in DPBS+1%BSA) was added to the wells, and the plates were incubated at room temp. for 1 hr.

5.   The plates were then washed with DPBS, and developed

35   with the TMB Microwell Substrate System (Kirkegaard and Perry Laboratories, Inc.)

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C.   RECOMBINANT P-selectin

    1.   Ninety six well flat bottom plates were coated overnight at 4°C with 100µl of P-selectin which had been shed into the serum free medium of a pool of 293 cell clones transfected with the recombinant P-selectin gene. This supernatant contained at least 8ng/ml of P-selectin and was coated onto COSTAR plates either undiluted or diluted 1:8 with media.

    2.   The plate was washed once with Dulbecco's PBS (DPBS) and blocked with 200µl DPBS containing 1% BSA for 30 min at room temperature.

    3.   The plate was then processed for the ELISA Assay according to steps 7-13 in Section A, Page 9.

D.   ISOTYPING ANALYSIS OF mABs to P-Selectin

    Isotype analysis of mu MAbs PB1.3 and PNB1.6 indicated that both monoclonal antibodies were of the mouse immunoglobulin subclass, IgG1. Isotyping was performed using the capture method of Boehringer Mannheim Mouse-Hybridoma-Subtyping Kit.

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Example 5

Detection of P-selectin

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    This example describes the detection of P-selectin by western blotting. Samples containing P-selectin were mixed with an equal volume of SDS-PAGE sample buffer (non-reducing), heated at 100°C, run on a Novex 8-16% gradient gel, and transferred electrophoretically to nitrocellulose. The nitrocellulose membrane was then blocked for at least 1 h in phosphate-buffered saline (PBS) +1% bovine serum albumin (BSA). The membrane was then incubated at room temperature for 1 h or longer with either tissue culture supernatant from hybridomas expressing the appropriate monoclonal antibody or purified antibody (5 µg/ml) in PBS+1%BSA. The membrane was then washed several times in PBS+1%BSA, and incubated for 1 h with a 1 to 1000 dilution of sheep anti-mouse IgG horseradish peroxidase conjugate (Sigma A-6782) in PBS+1%BSA. The membrane was then washed, and bands visualized with

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tetramethylbenzidine/membrane enhancer (Kirkegaard and Perry
Laboratories, Inc.)

For tissue culture supernatants from both mu MAbs
PB1.3 and PNB1.6 a protein was detected having a molecular
5   weight of 140Kd. This was shown in preparations of
solubilized platelet membranes as well as in purified P-
selectin.

Example 6
In Vivo Inhibition of Acute Inflammatory Injury by
10   Blocking P-selectin Antibody

This example provides data demonstrating the ability
15   of blocking P-selectin antibody of the invention to treat
acute lung injury. In this example, systemic complement
activation was produced by vascular infusion of the cobra
venom factor (CVF) into rats. Injury in this model develops
rapidly and is known to be dependent on toxic oxygen products
20   generated from neutrophils.

For these studies, the two monoclonal antibodies
described in Examples 2 and 3 were used. Of particular
importance for this study, the blocking P-selectin antibody mu
MAb PB1.3 also inhibited adhesion of thrombin-activated rat
25   platelets to human neutrophils (Fig. 1). These data suggest
that mu MAb PB1.3 recognizes a conserved functional P-selectin
epitope and that a blocking P-selectin antibody might be
useful in the treatment of other inflammatory injuries.

For the in vivo experiments 20 units CVF/kg body
30   weight were infused intravenously into 300 gm adult male Long-
Evans rats. This results in rapid intrapulmonary,
intravascular sequestration of blood neutrophils, with
resulting damage of interstitial capillary endothelial cells
at points of physical contact of endothelial cells and
35   neutrophils. When employed, mu MAbs PB1.3 and PNB1.6 were
infused intravenously in the amounts indicated together with
CVF, in total volume of 0.5 ml. Negative control animals
received 0.5 ml intravenous infusion of phosphate buffered
saline (PBS). In all cases the infusion material also
40   contained trace amounts of (^{125}I)-bovine serum albumin and

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homologous (^{51}Cr)-red blood cells (RBC). The parameters of
lung injury (leakage of albumin and extravasation of RBC) were
determined at 30 min. according to established techniques
Mulligan, et al., *J. Clin Invest.* 88:1396 (1991).

5   The results of these experiments are shown in Figs.
2A and 2B. Coinfusion of 200 μg PNB1.6 (the non-blocking
anti-P-selectin antibody) together with CVF failed to cause
any reduction in lung injury when compared to the values of
untreated CVF positive controls. Thus animals receiving CVF

10   PNB1.6 in PBS served as the reference positive control values.
When 100 μg mu MAb PB1.3 antibody was infused with the CVF
permeability was reduced by 19.2% (p=.002) and hemorrhage was
reduced by 37.5% (p=.001) (data now shown). When 200 μg mu
MAb PB1.3 was added, permeability was reduced by 50.8%

15   (p<.001) (Fig. 2A) and hemorrhage by 70.0% (p<.001) and 70.1%
(p<.001), respectively (Fig. 2B).
           Lungs from companion sets of animals were
homogenized sonicated and the myeloperoxidase activity (MPO)
measured in order to obtain an estimate of the neutrophil

20   content in lung. MPO was determined by the decomposition of
H_2O_2 in the presence of o-dianisidine according to standard
procedures, Warren et al. *J. Clin. Invest.* 84:1873 (1989). As
shown in Fig. 3A, treatment with 100, 200 or 400 μg mu MAb
PB1.3 reduced MPO content below the values in the reference

25   (untreated) positive control group by 26% (p=.024), 41%
(p<.001) and 50% (p<.001), respectively. In animals injected
with 200 μg of PNB1.6 there was no reduction in MPO content
(Fig. 3A), consistent with the inability of this antibody to
protect against CVF-induced lung injury. Thus, the protective

30   effects of the blocking antibody mu MAb PB1.3, correlate with
its ability to interfere with neutrophil accumulation in lung
tissue. Transmission electron microscopic examination of lung
sections confirmed that treatment with mu MAb PB1.3 resulted
in reduced accumulation of neutrophils within the pulmonary

35   interstitial capillaries, diminished adherence of neutrophils
to endothelial cells, and reduced evidence of damaged
endothelial cells (Fig. 3B).

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        In order to investigate lung expression of P-
selectin following intravenous injection of CVF, an additional
group of rats was infused with CVF and animals sacrificed at
times 0, 5, 10, 15, 20 and 60 min later. Lungs were inflated
5   with O.C.T., snap frozen, and sections obtained and examined
for presence of P-selectin by immunohistochemical techniques,
using mu MAb PB1.3. Little detectable reactivity in the
pulmonary vasculature was found at time 0, whereas staining
was clearly evident at 5 min and was increased at 15 and 20
10   min after infusion of CVF. The pattern of staining involved
pulmonary venules and septal areas in a pattern consistent
with staining of interstitial capillaries. At 60 min.
staining had largely disappeared (data not shown). Although
P-selectin is presumably present in intracellular storage
15   granules prior to CVF infusion, it is apparent that
mobilization of P-selectin to the surface of the endothelium
dramatically enhanced its reactivity with mu MAb PB1.3
antibody.

20   Example 7
                   Competitive Binding Assay
        Methods of isolation of platelets from human blood,
activation of isolated platelets with thrombin and isolation
of PMNs (neutrophils) are described in Example 1 above.
25   INCUBATION OF THROMBIN ACTIVATED PLATELETS WITH MONOCLONAL
ANTIBODIES TO P-SELECTIN.
        a.   Place 20 μl thrombin-activated platelets (2×10^8/ml)
             into each of 24 Eppendorf tubes (1.5 ml) in
             duplicate.
30   b.   To 8 of these tubes, add 20 μl of the IgG fraction
             purified from PNB1.6 at a concentration of 10 μg/ml
             in Tyrode-Hepes Buffer, pH 7.2 containing 1 mM
             $CaCl_2$. To a second row of 8 tubes, add 20 μl of the
             same IgG preparation at a concentration of 1 μg/ml.
35   To the third row of 8 tubes add 20 μl of the buffer
             alone.
        c.   Mix and stand at room temperature for 20 min.

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d.   To each row of 8 tubes add 20 μl of the IgG fraction purified from mu MAb PB1.3 at concentrations ranging from 10 μg/ml to 0.03 μg/ml.

e.   Mix and stand at room temperature for 20 min.

f.   Add 20 μl neutrophils at $3x10^6$/ml.

g.   Mix and stand at room temperature for 20 min.

h.   Evaluate adhesion microscopically as follows: Count 100 neutrophils in each sample. Score a cell as positive if it has bound 2 or more platelets, and as negative if it has bound less than 2. Calculate the percentage of positive cells.

As shown in Fig. 1, pretreatment of thrombin activated platelets with mu MAb PNB1.6 did not affect the ability of mu MAb PB1.3 to block their P-Selectin mediated adhesion to neutrophils.

Example 8
Characterization of Binding of Blocking P-selectin Antibodies

I(a)   Purification of Monoclonal Antibodies: The monoclonal antibodies PNB1-6 and 84/26 were isolated from tissue culture supernatants by passage over a column of protein G Sepharose 4 Fast Flow (Pharmacia). The column was washed extensively with phosphate-buffered saline (PBS), and bound antibody was eluted with 0.1M glycine-HCl pH 2.7 into tubes containing 0.2 to 0.5 volume 1M Tris, pH 8.8. The procedure used for isolating mu MAb PB1.3 was identical except that the antibody bound to protein G was eluted with 0.1M acetate-HCl pH 2.5 into tubes containing 0.3 volumes 2M Tris, pH 10. Antibody containing fractions were pooled, and dialyzed against several changes of PBS at 4°C.

(b)   Preparation of mu MAb PB1.3 Affinity Column: For use in the purification of P-selectin, mu MAb PB1.3 was coupled to tresyl activated agarose according to the manufacturer's affinity instructions (Schleicher and Schuell).

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(c) <u>Purification of P-Selectin</u>: to washed, outdated human
platelets, prepared as described above, was added 1/100th
volume 5 mg/ml leupeptin, and 1/100th volume 35 mg/ml
AEBSF (Calbiochem). After mixing, the platelets were
5   freeze-thawed three times in either a dry-ice/methanol
bath or liquid nitrogen, homogenized ten strokes with a
Dounce homogenizer, and centrifuged for 1 h at 35,000 rpm
at 4°C in a Beckman 70TI rotor. The pellet was
resuspended in Dulbecco's phosphate-buffered saline
10   (DPBS, Whittaker Bioproducts, which contains
approximately 0.9 uM CA^{++} and 0.5 mM Mg^{++}), and made 5 mM
in benzamidine-HCl, 100 uM in leupeptin, and 2% in Triton
X-100. The suspension was resuspended, and homogenized
10 strokes with a Dounce homogenizer. After a 1 h
15   incubation at 4°C, the solution was again centrifuged in
a Beckman 70TI rotor for 1 h at 4°C at 35,000 rpm. The
supernatant was passed over a column of mu MAb PB1.3-
agarose that had been equilibrated with DPBS+0.05% Rennex
30 (Accurate Chemical and Scientific Corp.). The column
20   was washed extensively with DPBS+0.05% Rennex 30, and
bound P-selectin was eluted with 0.1M triethylamine-HCL,
0.05% Rennex 30, pH 11.5, into tubes containing 0.1
volume 1 M phosphate, pH 6.8. Fractions containing P-
selectin were pooled, dialyzed against DPBS+0.05% Rennex
25   30, concentrated using either a Centricon 30 or
Centriprep 30 spin concentrator (Amicon), aliquoted, and
stored at -80°C.

(d) <u>Preparation of the Peptide CQNRYTDLVAIQNKNE (Cys-Gln-Asn-
Arg-Tyr-Thr-Asp-Leu-Val-Ala-Ile-Gln-Asn-Lys-Asn-Glu-NH$_2$)</u>:
30   The peptide was synthesized on an Applied Biosystems
(Foster City, CA) 430A peptide synthesizer using Fmoc
protected amino acids and 2-(1H-Benzotriazol-1-yl)-
1,1,3,3-tetramethyluronium hexafluorophosphate (HBATU)
esters for amino acid activation. Each amino acid was
35   routinely double coupled. Fmoc protected amino acids and
Hydorxybenzotriazole were purchased from Applied
Biosystems. All solvents were purchased from Burdick and
Jackson. HBTU was purchased from Richelieu

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Biotechnologies (St. Hyacinthe, Canada). Piperidine and
trifluoroacetic acid, acetic anhydride, thioanisole,
phenol and ethanedithiol were purchased from Sigma
Chemical Corporation.

5   Fmoc-Amide resin (Bachem Biosciences) was loaded into the
peptide synthesis reaction vessel and washed one time
with N-methylpyrolidone (NMP). The following operations
were then sequentially performed:

1.   The Fmoc protecting group was removed by
10   treatment of the resin bound amino acid with
25% piperidine in NMP.

2.   The resin was washed 5 times with NMP.

3.   A mixture containing N-α-Fmoc-L-Glutamic acid
γ-t-butylester, diisopropylethylamine, HBTU and
15   NMP was added to the reaction vessel and
allowed to react for 30 min, under vortex
agitation.

4.   The solvent was drained, and the resin was
washed three times with NMP.

20   5.   Steps (3) and (4) were repeated two more times.

6.   The resin was washed four more times with NMP.

Steps 1-6 were repeated for each amino acid of the peptide.
Following the final coupling cycle, the resin-bound peptide
25   was deprotected by reaction with 25% piperidine in NMP, washed
7 times with NMP, and washed 2 times with dichloromethane.
The resin was dried *in vacuo* for 24 h. The peptide was
cleaved from the resin by treatment with trifluoroacetic acid
containing 2.5% ethanedithiol, 5% thioanisole, 7.5% phenol,
30   and 5% water. The polystyrene resin was separated from the
trifluoroacetic acid solution by filtration. Trifluoroacetic
acid was removed by evaporation *in vacuo*. The crude peptide
was triturated with diethylether and dissolved in water. The
water was removed by lyophilization. The peptide was then
35   purified by reverse phase HPLC on a C_8 column (VYDAC) using a
gradient of acetonitrile, water, each containing 0.1% TFA as
modifier.

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    (e) <u>Biotinylation of Monoclonal Antibodies</u>: Antibodies were dialyzed against 0.1M sodium bicarbonate, pH 8.5. NHS-LC Biotin (Pierce) was added to 0.3 mg/ml. After 2 hrs at room temperature, antibodies were dialyzed against

5   several changes of PBS.

    (f) <u>Coating of Microtiter Plates for ELISA</u>: 96 well microtiter plates (Falcon Microtest III) were coated overnight at 4°C with 50 μl/well of 2 μg/ml affinity purified P-selectin in DPBS.

10   (g) <u>Western Blotting</u>: mu MAbs PB1.3, PNB1.6, and 84-26 all bind to a single band of 140 KD when platelets are dissolved in SDS-PAGE running buffer (unreduced), subjected to SDS-PAGE, and transferred to nitrocellulose (data not shown). We have also found that mu MAbs PB1.3

15   and PNB1.6 no longer recognize P-selectin on western blots when the samples have been reduced with β-mercaptoethanol.

II. <u>Effect of Chelating Divalent Cations on Binding of</u>

20 <u>Monoclonal Antibodies to P-Selectin</u>: Two microtiter plates were coated with affinity purified P-selectin as above. Plate 1 was blocked with 200 μl/well PBS+1% BSA, while plate 2 was blocked with 200 μl/well DPBS+1% BSA (DPBS contains Ca^{++} and Mg^{++}). After 1 hr, the plates were washed (all washes for

25 plate 1 were PBS, for plate 2, with DPBS. 25 μl of 25 mM EDTA in PBS+1% BSA was added to the wells of plate 1, and 25 μl of DPBS+1% BSA was added to plate 2. After 1 hr, 25 μl of dilutions of the appropriate antibody were added to the wells of either plate. Dilutions for plate 1 were made in PBS+1%

30 BSA, for plate 2 in DPBS+1% BSA. After 1 hr, the plates were washed, and 50 μl of a 1 to 1000 dilution of sheep anti-mouse IgG horseradish peroxidase conjugate (dilutions for plate 1 were made in PBS+1% BSA, for plate 2 in DPBS+1% BSA) was added. After 1 hr, the plates were washed, and 50 μl of the

35 peroxidase substrate TMB (3,3',5,5'-tetramethylbenzidine, Kirkegaard and Perry Laboratories, Inc.) was added. After the color had developed to the appropriate level, the substrate

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reaction was quenched by the addition of 25 μl of 1M
phosphoric acid and the absorbance at 450 nm was read.

    Fig. 4 shows the binding of the anti-P-selectin
monoclonal antibodies to P-selectin in the buffer DPBS, which
5   contains CA^{++} and Mg^{++}, and in PBS + 25 mM EDTA, a chelator of
divalent metal cations. The concentration of divalent cations
present in DPBS is more than sufficient to support neutrophil
adhesion to P-selectin (Geng et al., 1991), while the chelator
EDTA, at 25 mM, is more than sufficient to block neutrophil
10   adhesion to P-selectin. The binding of all of the monoclonal
antibodies to P-selectin is little affected by the presence of
EDTA, demonstrating the Ca^{++} is not required to be present for
binding to occur. Mu MAb PB1.3 is a blocking antibody, that
is, it is able to block the binding of neutrophils P-selectin,
15   for example on activated platelets. The PNB1.6 is a non-
blocking antibody. The ability of 84/26 to block the binding
of neutrophils to P-selectin has not yet been fully
characterized. In contrast, of the anti-P-selectin monoclonal
antibodies described by Geng et al., only the non-blocking
20   antibody S12 is able to bind in the absence of Ca^{++}.

III.   <u>Effect of the Peptide CQNRYTDLVAIQNKNE on the binding of</u>
<u>mu MAb PB1.3 to P-Selectin</u>: A microtiter plate was coated
with affinity purified P-selectin as above, blocked with 200
25   μl/well DPBS+1% BSA for 1 hr, and washed. Dilutions of mu MAb
PB1.3 in DPBS+1% BSA were mixed with either 0.35 mg/ml
CQNRYTDLVAIQNKNE in PBS, or as a control, PBS alone. After 1
hr, 50 μl of each antibody dilution was added to the
microtiter plate, and incubated for 1 hr. The plate was
30   washed with DPBS, and a 1 to 1000 dilution of sheep anti-mouse
IgG horseradish peroxidase conjugate in DPBS+1% BSA was added.
After 1 hr, the plate was washed, the substrate TMB was added,
and the color development was stopped and the plates read as
above.
35   Fig. 5 shows that the peptide CQNRYTDLVAIQNKNE,
homologous to residues 19-34 of the lectin domain of P-
selectin, has no effect on the binding of mu MAb PB1.3 to P-
selectin when the peptide is present at a concentration of

0.35 mg/ml. This distinguishes this particular monoclonal antibody from the monoclonal antibodies G1, G2, and G3, whose binding to P-selectin is partially or completely blocked by this peptide.

5

IV.   <u>Ability of Cytel Mu MAbs PB1.3, PNB1.6, and 84/26 to</u> <u>Block Binding of Each Other to P-Selectin</u>: Microtiter plates coated with affinity purified P-selectin as above were blocked for 1 h with DPBS+1% BSA, and washed. 25 µl of a 50 µg/ml

10   solution of mu MAbs PB1.3, PNB1.6, or 84/26 in DPBS+1% BSA were added to appropriate wells, and incubated for 1 hr. Then, 25 µl of a dilution (in DPBS+1% BSA) of biotinylated antibody was added. The plate was then incubated on a platform shaker for 1 hr. After washing, 50 µl of a 1 to 1000

15   dilution of a streptavidin horseradish peroxidase conjugate was added, and incubated for 1 hr. After washing with DPBS, the plate was developed with the substrate TMB as above.
      Fig. 6 shows the ability of the mu MAbs PB1.3, PNB1.6, and 84/26 to interfere with the binding of each other

20   to P-selectin. Fig. 6A shows that only mu MAb PNB1.6 is able to block the binding of biotinylated mu MAb PNB1.6 to P-selectin, demonstrating that mu MAbs PB1.3 and 84/26 must recognize different epitopes from mu MAbs PNB1.6. Likewise, In Fig. 6B, mu MAb PB1.3, but not mu MABs PNB1.6 or 84/26, is

25   able to block the binding of biotinylated PB1.3 to selectin, demonstrating that the other two monoclonals must recognize different epitopes. Fig. 6C, both mu MAbs 84/26 and PB1.3, but not mu MAb PNB1.6, are able to block the binding of biotinylated mu MAb 84/26 to P-selectin.

30

V.   <u>Blocking P-selectin antibodies do not inhibit thrombin-</u> <u>induced aggregation of human platelets</u>:
      This example demonstrates that the P-selectin antibodies, mu MAbs PB1.3 and PNB1.6 do not inhibit thrombin-

35   induced aggregation of platelets. Fresh human platelets were isolated as previously described and suspended in Tyrode-Hepes Buffer, pH 7.2 at a concentration of 2×10^8/ml.

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Platelet aggregometry was performed in a Lumi
aggregometer (Chrono-Log Corp.). For the assay, 0.45 ml of
the platelet suspension was placed in a standard siliconized
cuvette. To this was added 10 µl of the antibody or vehicle
5   control. After 5 min. at 37°C, 0.1 unit of thrombin (Human,
Sigma) was added and aggregation was recorded.

When platelet aggregation was measured under control
conditions, in the absence of antibody, an aggregation
response of 71 units was obtained. An equivalent extent of
10   aggregation was obtained in the presence of 10 µl of undiluted
culture supernatants from cells producing mu MAb PB1.3
antibody (Fig. 7). Different concentrations of mu MAb PB1.3
(1-100 µg/ml) were added to the platelet suspension. At no
concentration of mu MAb PB1.3 was platelet aggregation
15   different from the response determined in the absence of
antibody (Fig. 7).

Example 9
In vivo Protective Effects of Blocking P-Selectin Antibody,
20   _mu MAb PB1.3 in Feline Myocardial Ischemia and Reperfusion_

A model of myocardial ischemia and reperfusion
injury in cats was performed according to the methods of Tsao
et al. (Circulation 82:1402-1412 (1990)). Briefly, male cats
25   (2.5-3.5 Kg) were anesthetized with sodium pentobarbital (30
mg/Kg, i.v.). An intratracheal tube was inserted through a
mid-line incision and all cats were given intermittent
positive pressure ventilation by a Harvard small animal
respirator. A polyethylene catheter was inserted into the
30   external jugular vein and the right femoral vein was
cannulated and connected to a Statham P23Ac pressure
transducer for the measurement of arterial blood pressure. A
midline thoracotomy was performed, the pericardium was opened
and the heart was exposed. A 2-0 silk suture was carefully
35   placed around the left anterior descending artery (LAD) 10-12
mm from its origin. After a 30 min period of stabilization
myocardial ischemia was initiated by complete ligation of the
LAD for 1.5 h of ischemia followed by 4.5 h of reperfusion.
Blocking (mu MAb PB1.3) and non-blocking (mu MAb PNB1.6) anti-

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P-selectin antibodies were administered intravenously at a
dose of 1 mg/kg 10 min prior to the initiation of reperfusion.

5   The ischemic myocardium was determined as that
portion of tissue which did not stain with nitroblue
tetrazolium and was expressed as a percentage of the area at
risk. Area at risk was determined by reocclusion of the LAD
at the end of the reperfusion period followed by the injection
of Evans blue dye into the left atrium. The area of risk was,
10   therefore, determined by negative staining.
        Endothelial dependent relaxation of coronary artery
rings was determined by measuring the acetylcholine-induced
relaxation of rings previously contracted with the thromboxane
A2 mimetic U46619. Data are expressed as percentage
15   relaxation.
        Neutrophil accumulation in the myocardium was
measured as the myeloperoxidase activity of polytron
homogenized myocardial tissue.
        The results of these experiments are shown in
20   Fig. 8. In cats treated with the non-blocking anti-P-selectin
antibody mu MAb PNB1.6 the extent of myocardial ischemia was
33 ± 5% of the area at risk. In contrast, the extent of
ischemia in PB1.3 treated animals was significantly (P<0.01)
less at 15 ± 3% of the area at risk. Endothelial dependent
25   relaxation to acetylcholine was also significantly preserved
in ischemic-reperfused coronary arteries taken from cats
treated with mu MAb PB1.3 compared to mu MAb PNB1.6 (67 ± 6 vs
11 ± 3%, P<0.01). That these phenomena are attributable to a
reduction in myocardial leukocyte infiltration is suggested by
30   a significant (P<0.1) reduction in the number of PMNs in
myocardial tissue from mu MAb PB1.3-treated animals (64 ± 7
PMN/mm^2) compared with mu MAb PNB1.6 treated animals (136 ± 12
PMN/mm^2).

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Example 10
Production of Humanized Antibodies to P-Selectin

A. Cloning and Sequencing of Mu MAb PB1.3 Variable Regions

The isolation of mu MAb PB1.3 is described in

5   Example 2. Total RNA was isolated from hybridoma cells
producing mu MAb PB1.3. The cDNAs encoding the variable
regions of the heavy and light chains of mu MAb PB1.3 were
amplified by polymerase chain reaction (PCR). The entire
mouse variable region was amplified using a mixture of

10   degenerate primers that hybridized within the leader sequence
and a single primer that hybridized to the mouse constant
region near the V-C junction. *See* Jones & Bendig,
Bio/Technology 9:88-89 (1991) and *Bio/Technology* 9:579 (1991).
The PCR fragments were cloned and sequenced. The nucleotide

15   sequences and the corresponding amino acid sequences for the
variable regions of the heavy and light chains of mu MAb PB1.3
are given in Figs. 9 and 10, respectively.

B. Modelling the Structure of the Mu MAb PB1.3 Variable
20   Regions

A molecular model of the V_L and V_H regions of mu MAb
PB1.3 was built. The model was built on a Silicon Graphics
IRIS 4D workstation running under the UNIX operating system
and using the molecular modelling package QUANTA (Polygen

25   Corp., USA). The amino acid sequence of the mu MAb PB1.3
variable regions were compared with the sequences of
structurally-solved immunoglobulin variable regions. *See*
Tables 1 and 2.

C. Design of Reshaped MAb PB1.3 Variable Regions
30

Human variable regions were selected that have
framework regions most similar to those found in the mu MAb
PB1.3 variable regions. The human mAb DEN light chain
variable region and the human mAb 21/28'CL heavy chain

35   variable region were selected as frameworks to join the
corresponding complementarity determining regions (CDRs) of mu
MAb PB1.3. The human framework regions were modified using
the methodology described in *Monoclonal Antibodies, 2:*

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Applications in Clinical Oncology (Editor A. Epenetos:
Chapman & Hall, 1992, which is incorporated herein by
reference; *see* especially, Bendig et al., *The Humanization of
Mouse Monoclonal Antibodies by CDR-Grafting: Examples with
Anti-Viral and Anti-Tumor Cell Antibodies* contained therein,
which is incorporated herein by reference). The alignment of
amino acids of the variable regions leading to the design of
the reshaped humanized PB1.3 variable regions are given in
Tables 1 and 2.

Three versions of the heavy chain variable region
were modelled and were designated $CY1748RH_A$, $CY1748RH_B$, and
$CY1748RH_C$. The nucleotide sequences and corresponding amino
acid sequences of these variable regions and signal peptides
are given in Figs. 11, 12, and 13, respectively. In the case
of $CY1748RH_B$ the first two mouse residues conserved in
$CY1748RH_A$ (glutamic acid and alanine) were changed back to the
original human amino acids (glutamine and valine) found in the
donor human FR1. In the case of $CY1748RH_C$, computer modelling
suggested that the murine lysine at position 73 might form a
salt bridge with residue 55 (aspartic acid) in CDR2. If this
were the case, then the lysine would be important for the
stabilization of the H2 loop. The human threonine residue at
this position would be unable to form such a salt bridge.
Therefore, version $CY1748RH_C$ substitutes the murine lysine at
position 73 of $CY1748RH_A$. Version $CY1748RH_B$ contains the
highest number of human amino acid residues. A summary of the
amino acid differences among these three versions of the heavy
chain is given in Table 3.

Four versions of the light chain variable region
were modelled and were designated $CY1748RL_A$, $CY1748RL_B$,
$CY1748RL_C$, and $CY1748RL_D$. The nucleotide sequences and
corresponding amino acid sequences of these variable regions
and signal peptides are given in Figs. 14, 15, 16 and 17,
respectively. Computer modelling suggested that the murine
residue glutamic acid at position 60 might be important in P-
selectin binding. This residue was incorporated in version
$CY1748LR_A$ but replaced with the original human framework
residue serine in version $CY1748LR_B$. The murine aspartic acid

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residue might have a charge interaction with residue 24 in loop 1 (L1), therefore CY1748LR$_C$ is identical to CY1748LR$_A$ except for the replacement of the mouse residue aspartic acid at position 70 for the human framework amino acid glutamic

5   acid. CY1748LR$_D$ incorporates both these amino acid replacements and of the four versions contains the greatest number of human amino acid residues. A summary of the amino acid differences among these four versions of the light chain is given in Table 4.

10

D.   Subcloning of cDNAs Encoding Heavy and Light Chain Variable Regions Into Expression Vectors
          (1)   Construction of Expression Vectors
               (a) pHCMV-1748RL-KR-neo

15   The expression vector contains the human cytomegalovirus (HCMV) enhancer and promoter to drive transcription of the recombinant immunoglobulin light chain, the coding sequences of the bacterial neo gene to act as a dominant selectable marker during stable transformation, and

20   the SV40 origin of replication to give high levels of transient expression in cos cells. To construct the vector, a HindIII-SacII fragment from a pUC8 vector containing the PstI-m fragment of HCMV (Boshart et al., Cell 41:521-530 (1985)) was converted to an EcoRI-HindIII fragment using appropriate

25   adaptor oligonucleotides. In a three-way ligation, the 1.2 kb EcoRI-HindIII fragment containing the HCMV enhancer-promoter was ligated to a 5.05 kb BamH-EcoRI fragment from pSV2neo (Southern and Berg, J. Mol. App. Gener. 1:327-341 (1982)) and a 0.5 kb HindIII-BamHI fragment containing the V$_L$lys kappa

30   light variable region (Foote and Winter, J. Mol. Biol. 224:487-499 (1992)). The HindIII site in pSV2neo had previously been removed by filling-in with Klenow polymerase producing a vector designated pHCMV-V$_L$lys-neo.
               A ¯2.6 kb EcoRI fragment of the genomic human kappa

35   region was cloned (Rabbitts et al., Curr. Top. Microbiol. Immunol. 113:166-171 (1984)) and subcloned into a pUC vector, pUCKR17 (Mengle-Gaw, EMBO J. 6:1959-1965 (1987)). BamHI sites were added to both ends of the fragment using EcoRI-BamHI

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adapters. This fragment was then inserted into the BamHI site
of pHCMV-V$_L$lys-neo, in the appropriate orientation, to create
pHCMV-V$_L$lys-K$_R$-neo. To facilitate insertion of reshaped human
variable regions, the BamHI site on the 3'-side of the human

5   kappa constant region was removed by filling-in with Klenow
polymerase. In the resulting vector, designated pHCMV-1748RL-
KR-neo, the HindIII-BamHI fragment containing the V$_L$lys is
easily replaced with the VL of a reshaped antibody.

10

(b) pHCMV-1748-γ1C-neo

           The construction of this vector began with the same
three way ligation as described above for generation of pHCMV-
V$_L$lys-neo above save for the insertion of a 0.7 kb HindIII-

15   BamHI fragment containing the V$_H$lys heavy variable region
(Verhoeyen et al., *Science* 239:1534-1536 (1988)); Foote and
Winter, *supra*) in place the 0.5 kb HindIII-BamHI fragment
containing the V$_L$lys kappa light chain. The cDNA coding for
human γ1 constant region was inserted into the BamHI site to

20   produce a vector designated pHCMV-V$_H$lys-neo. cDNA coding for
human γ1 constant region was cloned from a human cell line
secreting a human γ1 antibody by PCR amplification. BamHI
sites were created at each end of the cDNA, and a splice
acceptor site and a 65bp intron sequence were introduced at

25   the 5-end of the cDNA sequence. The BamHI fragment (1176bp)
containing the human γ1 cDNA plus the splice acceptor site and
intron sequence was then cloned into the expression vector.
As with the light chain expression vector, the BamHI site at
the 3'-side of the human γ1 constant region was removed by

30   filling-in with Klenow polymerase. With this vector,
designated pHCMV-1748-γ1C-neo, the HindIII-BamHI fragment
containing the unwanted V$_H$lys is easily replaced with the V$_H$
of a reshaped antibody.

35   ### (2)   Insertion of Immunoglobulin Coding Sequences

           The cDNAs encoding the heavy chain variable region
of mu MAb PB1.3 and the three humanized versions were inserted
into the expression vector HCMV-γ1C-neo by standard techniques

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generating expression plasmids HCMV-1747CH-γ1C-neo, HCMV-
1748RH$_A$-γ1C-neo, HCMV-1748RH$_B$-γ1C-neo, and HCMV-1748RH$_C$-γ1C-
neo. The nucleotide sequence of the human IgG1 constant
region cDNA and corresponding protein sequence is given in
5   Ellison et al., *Nuc. Acids Res.*, 10:4071-4079 (1982). The
cDNAs encoding the light chain variable regions of
CY1747(PB1.3) and the four humanized versions were inserted
into the expression vector HCMV-KR-neo generating plasmids
HCMV-1747CL-KR-neo, HCMV-1748RL$_A$-KR-neo, etc. The nucleotide
10   sequence of the human kappa constant region gene and
corresponding protein sequence is shown in Hieter et al., *Cell*
22:197-207 (1980) (incorporated by reference in its entirety
for all purposes) Schematic maps of the heavy chain and
light chain expression vectors are given in Figs. 18 and 19.
15   By coexpressing appropriate combinations of heavy and light
chains, several antibodies can be expressed. For example,
coexpression of HCMV-1748RH$_A$-γ1C-neo and HCMV-1748RL$_A$-KR-neo
gives rise to the reshaped MAb PB1.3 (H$_A$/L$_A$). Coexpression of
HCMV-1747CH-γ1C-neo and HCMV-1747CL-KR-neo gives rise to the
20   chi MAb PB1.3 (i.e., chimeric antibody comprising murine
variable domains and human constant domains).

E.   Electroporation of COS Cells.

            The DNA constructions were tested in COS cells for
25   the transient expression of chi MAb PB1.3 and various reshaped
versions of MAb PB1.3 antibodies. DNA was introduced into the
COS cells by electroporation using the Gene Pulser apparatus
(Biorad). COS cells were trypsinized and washed once in
phosphate buffered saline (PBS). DNA (10 μg of each heavy
30   chain plasmid and appropriate light chain plasmid) and a 0.8
ml aliquot of 1 x 10^7 cells/ml in PBS were placed in a sterile
Gene Pulser Cuvette (Biorad, 0.4 cm gap). A pulse was
delivered at 1900 volts, 25 microfarads capacitance. After a
10 minute recovery period at ambient temperature, the
35   electroporated cells were added to 20 ml of DMEM media
(GIBCO/BRL) containing 10% fetal bovine serum. After a 48 h
incubation, the medium was collected, centrifuged to remove

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cellular debris, and stored under sterile conditions at 4°C
for short periods of time.

Example 11:   Analysis of Humanized Antibodies

5   Media from the transfected COS cells were assayed by
ELISA to quantify levels of human antibody produced and
analyze capacity to bind to recombinant soluble P-selectin
(rsP-selectin). Immulon microtiter plates (Dynatech, #011-
010-3355) were coated with 100 μl/well of a solution of goat
10   anti-human IgG (whole molecule, Sigma #I-1886) to a final
concentration of 12.5 μg/ml or a solution of rsP-selectin (1.2
mg/ml) diluted in Dulbecco's phosphate buffered saline (DPBS).
Plates were coated overnight at 4°C or for 2 h at 37°C. The
plates were then washed three times with DPBS. The plates
15   were then blocked with 400 μl/well of DPBS + 1% bovine serum
albumin (BSA, Sigma, #A-7888) for one h or longer at room
temperature. The plates were then washed three times with
DPBS.
              Serial dilutions of the monoclonal antibodies in
20   DPBS + 1% BSA were added to the plates (100 μl/well), and the
plates were incubated for 1 h at room temperature. The plates
were then washed three times with DPBS. 100 μl/well of a
second antibody (goat anti-human IgG peroxidase conjugate,
Sigma #A-8867) diluted 1 to 1000 in DPBS + 1% BSA was added to
25   the plates, and the plates were incubated for one h at room
temperature. The plates were then washed three times with
DPBS. 100 μl/well of tetramethylbenzidine peroxidase
substrate/H_2O_2 (TMB, Kirkegaard and Perry Laboratories #50-76-
00) was added to the plates, and the color was allowed to
30   develop for an appropriate period of time (usually 3 to 15
min), and the reaction was quenched by the addition of 100
μl/well of 1-M phosphoric acid. The plates were read at 450
nm in a Titertek Multiskan MCC/340.
              The concentration of the antibodies produced in all
35   COS cell supernatants was determined by comparing the IgG
ELISA results to a human IgG1 antibody (kappa light chain,
Sigma #I-3889) of known concentration.

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P-selectin binding was plotted as optical density
against the concentration of IgG. Binding curves of chi PB1.3
and various reshaped versions of reshaped MAb PB1.3 are given
in Figs. 20, 21, and 22. The objective was to select a
5   humanized antibody showing substantially indistinguishable
binding characteristics from chi MAb PB1.3. If more than one
humanized antibodies showed such characteristics, the one
containing the most human amino acid residues (or the fewest
murine amino acids) would be preferred, because this version
10   would be least likely to induce a HAMA response in clinical
use. All four of the reshaped light chains coexpressed with
heavy chain CY1748RH$_A$ generated antibodies that bound to rsP-
selectin as well as the chi MAb PB1.3. Of these, light chain
variable region CY1748LR$_D$ was selected for further
15   experiments, because it contains the most human amino acid
residues. The heavy chain variable regions CY1748RH$_A$ and
CY1748RH$_B$ when coexpressed with light chain variable regions
CY1748RL$_A$ and CY1748RL$_D$ also generated antibodies that bound
to rsP-selectin to the same extent as chi MAb PB1.3 (Fig. 22).
20   Of these heavy chains, the CY1748RH$_B$ heavy chain is preferable
because it contains the most human amino acid residues. Thus,
a preferred humanized antibody is formed from light chain D
and heavy chain B and is designated reshaped MAb PB1.3
(H_B/L_D).

25

Example 12
Stable Expression of Reshaped MAb PB1.3 (H_B/L_D) in CHO Cells
A.   Introduction of Humanized Immunoglobulin Chains in CHO cells
30   ### (1)   Construction of Vectors
                   #### (a) pHCMV-1748RH-γ1C-dhfr
         This vector is identical to pHCMV-1748RH-γ1C-neo
described in Example 11 except that the neo gene is replaced
by the dihydrofolate reductase (dhrf) gene linked to a
35   defective SV40 promoter-enhancer sequence. In order to remove
the enhancer sequence from the SV40 early promoter, the
plasmid DNA, pSV2-dhfr (Subramani et al., Mol. Cell. Biol.
1:854-864 (1981)) was digested with SphI and PvuII, filled-in

with Klenow polymerase, and self-ligated to yield pSV2-dhfr-
ΔE. A ¯3.7 kb EcoRI fragment containing the HCMV promoter,
the $V_H$lys heavy chain variable region, and a genomic DNA clone
of the human γ1 constant region was excised from the plasmid
5   pHCMV-$V_H$lys-γ1-neo with EcoRI. This fragment was ligated to
EcoRI-digested pSV2-dhfr-ΔE to create pHCMV-$V_H$lys-γ1-dhfr.
The genomic clone of the γ1 constant region was replaced with
a BamHI fragment containing the cDNA clone of the human γ1
constant region-described in Example 11. The BamHI site on
10   the 3'-side of the human γ1 constant region was then removed
by filling in with Klenow polymerase as in Example 11. The
resulting vector was designated pHCMV-174RH-γC-dhfr. The
HindIII-BamHI fragment of this vector containing the unwanted
$V_H$lys is easily replaced with the V_H of a reshaped antibody.
15

(b)   pHCMV-1748RH-γ4-dhfr

This vector is identical to pHCMV-1748RH-γ1C-dhfr
described above, but for the replacement of the cDNA clone of
the human γ1 constant region with a genomic clone of the human
20   γ4 constant region. To construct the vector, a ¯ 7.0 HindIII
fragment of DNA containing the genomic clone of the human γ4
constant region (Brüggeman et al., *J. Exp. Med.* 166:1351-1361
(1987)) was subcloned into the HindIII site of pUC19 to create
a plasmid called 428D. Because the human γ4 fragment already
25   had a BamHI site near its 3-'end, the subclone was inserted
into pUC19 in the orientation that placed this site distal to
the BamHI site in the polylinker of pUC19. The 7.0 kb human
γ4 fragment was excised from 428D using BamHI and ligated into
pHCMV-$V_H$lys-neo to create the plasmid pHCMV-$V_H$lys-γ4-neo.
30   The final plasmid was constructed by a three-way
ligation of a 5.4 kb BamHI-HindIII fragment containing the
HCMV enhancer-promoter and the pSV2-dhfr-ΔE plasmid sequence,
a 0.5 kb HindIII-BamHI fragment containing the VH of reshaped
human 1748 antibody and the 7.0 kb BamHI-BamHI fragment
35   containing the genomic clone of the human γ4 constant region.

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(2)   Insertion of Immunoglobulin Coding Sequences

The cDNA encoding 1748RH$_A$ heavy chain variable region was cloned into expression vector HCMV-1C-dhfr generating HCMV-1748RH$_A$-1C-dhfr.   The cDNA encoding 1748RH$_B$ was cloned into expression vectors HCMV-1C-dhfr and HCMV-4C-dhfr generating the plasmids HCMV-1748RH$_B$-1C-dhfr and HCMV-1748RH$_B$-4C-dhfr, respectively.   The nucleotide sequence of the human IgG4 constant region gene and corresponding amino acid sequence is given by Ellison et al., *DNA*, 1:11-18 (1981).   The vector HCMV-1C-dhfr expresses an IgG1 isotype antibody and vector HCMV-γ4C-dhfr expresses an IgG4 isotype antibody when they are each coexpressed with the kappa light chain vector. The schematic maps of the dhfr-expression vectors are given in Figs. 23 and 24, respectively.

CHO dhfr-deficient cells were grown in αMEM (+ nucleosides, GIBCO/BRL) and 10% fetal bovine serum. Plasmid DNA was introduced into the COS cells by electroporation using the Gene Pulser apparatus. CHO cells were trypsinized and washed once in phosphate buffered saline (PBS).   DNA (10 μg of each heavy chain plasmid and appropriate light chain plasmid) and a 0.8 ml aliquot of 1 x 10^7 cells/ml in PBS were placed in a sterile Gene Pulser Cuvette (0.4 cm gap).   A pulse was delivered at 1900 volts, 25 microfarads capacitance. After a 10 minute recovery period at room temperature, the electroporated cells were added to 20 ml of αMEM (+ nucleosides)/10% FBS. After a 24-48 h incubation cells were trypsinized and plated into 100 mm dishes in αMEM (- nucleosides)/10% dialyzed FBS (to select for the expression of the dhfr-containing plasmid) supplemented with 500 μg/ml G418 (GIBCO/BRL, to select for the expression of the neo-containing plasmid). Media was changed every 3-4 days until colonies emerged. Single clones were isolated via cloning cylinders, expanded and analyzed for IgG production via ELISA.

Three sets of DNAs were introduced into the CHO dhfr-deficient cells: 1) HCMV-1748RH$_A$-γ1C-dhfr and HCMV-1748RL$_A$-KR-neo expressing reshaped MAb PB1.3 (H$_A$/L$_A$), IgG1 isotype; 2)HCMV-1748RH$_B$-γ1C-dhfr and HCMV-1748RL$_D$-KR-neo expressing reshaped MAB PB1.3 (H$_B$/L$_D$) IgG1 isotype; and HCMV-

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1748RH$_B$-γ4C-dhfr and HCMV-1748RL$_D$-KR-neo expressing a reshaped
MAb PB1.3 (H$_B$/L$_D$), IgG4 isotype.

CHO cells were expanded and seeded into 10-chamber
(6000 cm^2 total surface area) Nunc cell factories. The medium

5   was harvested at 72 h and passed over a protein-A Sepharose
Fast Flow (Pharmacia) column. The column was washed and
bovine IgG was eluted at pH 4.5. The humanized antibodies
were eluted at pH 3.5, dialyzed in PBS or 20 mM acetate, 0.15
M NaCl, pH 5.5. Antibody concentration was determined

10   spectrophotometrically and confirmed by an ELISA for human
IgG. (A purified human IgG4 antibody (kappa, Sigma #I-4639)
was used as the control for mu MAb PB1.3 (H$_B$/L$_D$) IgG4
isotype.) Purity was confirmed by SDS-PAGE.

15   B.   Competitive Binding Assay
The relative binding affinities of the various
versions of reshaped MAb PB1.3 to P-selectin were determined
by competitive ELISA assay. The assay measures the
concentration of unlabelled antibody that inhibits by 50% the

20   binding of labelled mu MAb PB1.3 to P-selectin.
Two ml of purified MAb PB1.3 (1 mg/ml) was mixed
with 100.8 μl of NHS-LC-biotin (1 mg/ml, Pierce) and incubated
overnight at 4°C. The 2.1 ml sample was loaded on a NAP 25
column (size exclusion column, Pharmacia). The column was

25   eluted with 0.3 ml fractions with PBS. Each fraction was read
at 280 nm. The highest O.D.280 nm fractions were pooled. The
biotinylated-MAb PB1.3 was titered against rsP-selectin to
select an appropriate concentration to use for the competitive
binding assay.

30   A 96-well plate (Microtest III, Falcon #3912) was
coated with 50 μl/well of rsP-selectin (diluted to 2 μg/ml in
DPBS) overnight at 4°C or 90 min at 37°C. The plate was
inverted and residual fluid was tapped away.
The coated plate and an additional blank plate were

35   blocked with 200 μl/well of DPBS + 1% bovine serum albumin
(BSA, Sigma #A-7888), for 1 h at room temperature. The blank
plate was inverted and the residual blocking fluid was tapped
away. The rsP-selectin coated plate was allow to continue

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blocking during the antibody dilution period. Serially
diluted sample antibodies (in DPBS + 1% BSA) were added to the
blank plate at 50 μl/well. The initial concentrations of the
antibodies were 100 μg/ml. Biotinylated-PB1.3 (diluted to 0.3

5   μg/ml in DPBS + 1% BSA) was added to the sample antibodies in
the blank plate, 50 μl/well, and mixed by pipetting up and
down. The rsP-selectin coated plate was washed three times
with DPBS. The sample antibody/biotinylated
-PB1.3 mixture was pipetted into the plate coated with rsP-

10   selectin, 50 μl/well, and allowed to incubate for one h at
room temperature. The plate was then washed three times with
DPBS.

Streptavidin horse radish peroxidase conjugate
(Pierce #21124) diluted 1:2000 in DPBS +1% BSA was added to

15   the plate, 50 μl/well and allowed to incubate for 1 h at room
temperature. The plate was then washed three times with DPBS.

50 μl/well of tetramethylbenzidine peroxidase
substrate/H_2O_2 (TMB, Kirkegaard and Perry Laboratories #50-76-
00) was added to the plates, and the color was allowed to

20   develop for an appropriate period of time (usually 3 to 15
min), and the reaction was quenched by the addition of 50
μl/well 1 M phosphoric acid. The plates were read at 450 nm
in a Titertek Multiskan MCC/340.

Apparent binding affinity is expressed as the

25   concentration of antibody that binds rsP-selectin at one-half
maximum optical density at 450 nm. The ratio of
concentrations at one-half maximum binding of the sample
antibody to that of the control (unlabelled PB1.3) antibody
provides an estimate of the relative binding affinity of a

30   test antibody to rP-selectin.

Results from two typical competitive binding
experiments are given in Figs. 25 and 26. In the first
experiment, twice as much reshaped MAb PB1.3 (H_AL_A), IgG1
isotype (2.0 μg/ml) as PB1.3 (1.0 μg/ml) is needed to inhibit

35   the binding of labelled PB1.3 to 50% of maximum. Therefore
this humanized antibody binds rsP-selectin 50% as well as
PB1.3. Similarly, in the second experiment PB1.3/(H_BL_D) (IgG4
isotype) binds to rsP-selectin 50% as well as PB1.3.

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Example 13
Increasing Antibody Production in CHO Cells Via DHFR Amplification

5   CHO cells were plated into flasks and allowed to
reach confluence before changing media (8 ml for a T-25 cm^2
flask or 20 ml for a T-75cm^2 flask). After incubating the
cells for 48-72 h the media was harvested and assayed for IgG
production by ELISA. The cells were trypsinized and counted.
10   Antibody production rate was expressed as the amount of
antibody in micrograms secreted by one million cells in a 24 h
period.

          The three to six highest-producing clones were
amplified separately, and the remaining seven best clones were
15   pooled and amplified. Cells were plated at a density of 1 x
10^5 cells/100 mm dish in selection media. For the first round
of amplification cells were plated in three sets of dishes
containing αMEM(- nucleosides)/10% dialyzed FBS supplemented
with 500 μg/ml G418 and either 10, 20 or 50 nM methotrexate
20   (Sigma #A-6770). Cultures were fed every four days. At 10-14
days single colonies were isolated by cloning cylinders,
expanded, and assayed for IgG production by ELISA. Additional
rounds of amplification were performed on single clones or
pools of clones at methotrexate concentrations 5-10 times the
25   initial methotrexate concentration.

          Table 5 provides a summary of the antibody
production for the CHO cells expressing three humanized
versions of PB1.3. One amplified clone expressing reshaped
MAb PB1.3 (H$_B$L$_D$) (IgG4 isotype) arose from a pool of clones
30   that expressed less than 0.2 μg/10^6 cells/day, amplified to
2.0 μg/10^6 cells/day in the first round, and reached 33.0
μg/10^6 cells/day after the third round. This clone was
selected to be grown for large scale production of reshaped
MAb PB1.3.

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Example 14

Bioreactor Run

160-g collagen-coated microcarrier beads (150-200
microns, JRH Biosciences #60142-100) were hydrated in 800-ml
5   of sterile water for 30 min and the water decanted. The beads
were resuspended in 800-ml water and autoclaved for 30 min.
After cooling to room temperature the beads were washed twice
with sterile serum free αMEM(-nucleosides) and resuspended in
1-1 complete media αMEM(-nucleosides)/5% dialyzed FBS.

10   The cell line CHO-48B4, which expresses reshaped MAb
PB1.3 (H_BL_D) (IgG4 isotype) at 33.0 μg/10^6 cells/day, was
expanded into 36 T-225 cm^2 in αMEM(-nucleosides)/10% dialyzed
FBS/500 nm methotrexate. When the cells reached confluence
they were trypsinized and resuspended in 2-1 complete media
15   (no methotrexate) at a final concentration of 5.0 - 8.0 x 10^4
cells/ml.

1-1 beads, 2-1 cell suspension, and 2-1 fresh
complete media were aseptically added to the 10-1 autoclaved
vessel of a Proteus 2000 Bioreactor (Wheaton). The headspace
20   was continuously perfused with air and 5% CO_2. Cells were
allowed to attach to the microcarriers by programming the
Proteus to stir the cell/microcarrier suspension at 70 rpm for
1 min and 0 rpm for 59 min. After a total of 48 cycles, 5-1
media was added to the bioreactor, which was programmed to
25   maintain the suspension at 37°C, pH at 7.0, dissolved oxygen
at 30% that of air, and stirred continuously at 50 rpm.

After 3 days the bioreactor was programmed to
replace complete media at 5-1/day utilizing a gravity filter
attached to the harvest port (to leave microcarriers in the
30   bioreactor). The harvested media was stored at 4°C until
processing. The bioreactor was run for 5-7 days until
accumulated cell debris clouded the media harvest (due to
aeration damage).

The media harvest was clarified by passing though a
35   Millipak-60 0.45 micron filter (Millipore). 20-1 filtered
supernatant was passed over a column (2.5 x 12 cm) of protein-
A Sepharose Fast Flow (Pharmacia) at a flow rate of 0.55 1/hr.
After all of the supernatant had passed through the column,

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the column was washed with 1.5-1 PBS (BioWhittaker, #17-516Y).
The column was pre-eluted with 8 x 40-ml 0.2-M sodium acetate,
pH 4.5, and eluted with 8 x 40-ml 0.2-M glycine, 0.5-M NaCL,
pH 3.5, both into tubes containing 10 ml 2-M Tris-HCl, pH 8.0.

5   The single fraction with the highest absorbance at 280 nm was
dialyzed into 20-mM acetate, 0.15-M NaCl, pH 5.5, and stored
at 4°C.

        200-mg antibody was harvested per day for 5-7 days.
The purification yield was approximately 50%. Therefore, one

10   bioreactor run yields 500-700 mg purified antibody.

                        <u>Example 15</u>
        <u>The Effect of Mu MAb PB1.3 on Tissue Injury Produced by
        Ischemia Reperfusion Following Replantation</u>

15   <u>of the Rabbit Ear</u>

                New Zealand White rabbits were anesthetized with a
combination of ketamine and xylazine followed by infiltration
of lidocaine into the base of the ear. The left ear was

20   partially amputated leaving the central artery, vein and a
bridge of cartilage intact. All nerves were divided to render
the ear totally anesthetic. The ear was then reattached and a
microvascular clip was placed across the artery to produce
total ischemia. Rabbits were kept in a room maintained at

25   23.5°C for 6 h then the microvascular clamp was removed and
the ear allowed to reperfuse. Treatment was administered
immediately prior to reperfusion with either mu MAb PB1.3(2
mg/kg) or with either saline or an isotype (IgG1) matched
murine monoclonal antibody designated PNB1.6 (2 mg/kg). Ear

30   volume was measured daily for 7 days by water displacement to
quantify tissue edema. On day 7 necrosis was estimated as a
percentage of total surface area.

                A significant increase in ear volume was determined
in control animals administered either saline or PNB1.6

35   following reperfusion of the ears (Fig. 27). Because no
differences between the two control groups were noted, their
data have been combined. An increase in edema was also seen
in animals treated with MAb PB1.3, the magnitude of the
increase was significantly less than that observed in the

40   control group at all time points measured. The extent of

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necrosis (Fig. 28), measured on day 7, was also significantly reduced in mu MAb PB1.3-treated animals compared with control animals.

5   Thus, ischemia followed by reperfusion of the replanted rabbit ear produces an edematous response initially followed by extensive tissue necrosis. These two manifestations of tissue injury are substantially and significantly reduced in animals pre-treated with the anti-P-selectin antibody mu MAb PB1.3.

10

Example 16

The Effect of Mu MAb PB1.3 on Vascular Patency Following Ischemia and Reperfusion of the Canine Skeletal Muscle

15   This experiment utilizes a model of ischemia and reperfusion of the isolated canine gracilis muscle. One aspect of the reperfusion injury is a phenomenon referred to as the "no-reflow" phenomenon in which blood flow through the microcirculation of a previously ischemic tissue is absent or
20   severely reduced following reperfusion.

An isolated canine gracilis muscle preparation was utilized as described previously (Carden et al. *Circ. Res.* 66:1436-1444 (1990)). Briefly, adult mongrel dogs were anesthetized with pentobarbital sodium. The skin overlying
25   the gracilis muscle was divided and the muscle was freed from connective tissue by blunt dissection. The obturator nerve was sectioned. The proximal caudal artery and vein were cannulated and all other collateral vessels ligated. The femoral artery cannula was connected to a constant flow
30   perfusion pump and the outflow from the femoral vein drained in to a reperfusion reservoir. Arterial and venous pressures were monitored via side branches and blood flow was adjusted to maintain perfusion pressure at 100 mm Hg. Vascular isolation was assumed complete if arterial perfusion pressure
35   decreased to less than 20 mm Hg when the perfusion pump was switched off.

Microvascular patency was assessed by perfusion of the isolated gracilis muscle with contrast media (india ink) at the end of the experimental protocol. Computerized video

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imaging was used to quantitate the number of ink-containing
microvessels (< 10 μm diameter) per muscle fiber in histologic
sections obtained from isolated canine gracilis muscles
subjected to 4.5 h of continuous perfusion, 4 h of ischemia
5   followed by 0.5 h of reperfusion or ischemia reperfusion in
the presence of the anti-P-selectin antibody mu MAb PB1.3 at a
concentration of 40 μg/ml in the perfusate.

        The ischemia and reperfusion protocol reduced the
number of patent microvessels to 36 $\pm$ 3 % of the value
10   determined in control animals. When mu MAb PB1.3 was included
in the perfusate the number of patent microvessels following
ischemia and reperfusion was 119 $\pm$ 18 % of control.

        Thus, the anti-P-selectin antibody, mu MAb PB1.3,
completely prevented the development of the "no-reflow"
15   phenomenon, determined by the number of patent microvessels,
in the ischemic and reperfused canine gracilis muscle.

Example 17
The Effect of Mu MAb PB1.3 on Leukocyte Endothelial Cell
20   ### Interactions Induced by the Degranulation of Tissue Mast Cells

I. Methods
    a) Intravital microscopy
        Male Wistar rats (200-250g) were obtained from
25   Harlan Sprague Dawley, Indianapolis, and were fasted for 12-24
hrs before the surgical procedure. Surgical anaesthesia was
induced by an intramuscular injection of ketamine/rompun/
acepromazine. A catheter was placed in the left jugular vein,
and a tracheostomy was performed to facilitate spontaneous
30   breathing. The abdomen was shaved and washed, and a 3/4 inch
long incision was made through the midline into the peritoneal
cavity, taking care to avoid any bleeding. The rat was placed
on a microscope stage specifically designed for the intravital
microscopy procedure. A well-vascularized posterior loop of
35   the ileum was exteriorized, and the mesentery draped over a
viewing pedestal heated to 37°C. Throughout the experiment,
the mesentery was superfused at 2 ml/min with a heated
bicarbonate-buffered saline solution (37°C, pH 7.4).
Additionally, small pieces of Saran-Wrap (previously soaked in

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alcohol and rinsed in saline) were placed over the ileum and
mesenteric areas peripheral to the viewed area, in order to
keep these areas moist. The microcirculatory beds were
observed through an intravital microscope (Mikron Instruments,
5   San Diego) equipped with 10x oculars and a 20x objective
(water immersion, numerical aperture 0.4). A high-resolution
video system, consisting of a video camera (Hitachi color CCD)
clamped onto the microscope, a video timer (American Video
Equipment), a VCR (Sony SVO-9500MD) and a monitor (Sony
10   Trinitron), permitted video recordings of the microscope
image. In each mesentery preparation, 3 postcapillary venular
segments of approximately 180 μm length were selected for
repeated recordings according to the following criteria: (1) A
true postcapillary venule receiving blood from confluent
15   capillaries, diameter 15-30 μm; (2) Brisk blood flow, so that
individual red blood cells could not be distinguished;
(3) Some baseline rolling of leukocytes (less than 8
leukocytes present at any given time in the vascular segment),
but no firmly adhering leukocytes. A leukocyte was defined as
20   being firmly adhering if it stayed immobile at the vessel wall
for more than 30 seconds.

          b) Experimental protocol
               One-minute video recordings of the baseline
25   leukocyte interaction in the selected venules were made at 20
and 25 min after completion of the surgical procedure. All
treatments were administered intravenously before the second
baseline recording. At 30 min after surgery, infusion of
compound 48/80 (Sigma, catalog #C4257) into the superfusion
30   buffer was begun, and continued throughout the rest of the
experiment. This yielded a final concentration of 10 μg/ml at
the mesentery, and was done in order to induce mast cell
degranulation. Venules were recorded again at 20 and 30 min
after the beginning of 48/80 application. The extent of
35   leukocyte-endothelial cell interactions was determined at the
end of the experiment by analyzing the recorded video tapes.
The number of clearly visible leukocytes (i.e., cells
interacting with the vessel wall) was determined from frames

frozen at exactly 0, 15, 30, 45, and 60 seconds of the
recorded sequence, and the mean from these five determinations
was calculated. Hence, counted leukocytes included rolling
cells as well as those firmly adherent.

5

    c) <u>Statistics</u>

        Each experimental group contained 5-6 animals with
2-3 venules from each animal. The mean and its standard error
is given. Significant differences among group means were
10   tested through analysis of variance followed by the Tukey-
Kramer's HSD test. Differences before and after treatments in
individual venules were studied by paired t-tests. All tests
were run using JMP software running on a Macintosh IIsi, and
significance was accepted at $p < 0.05$.

15

II. <u>Results</u>

        Topical application of compound 48/80 to the rat
mesentery resulted in visible degranulation of more than 90%
of the mast cells, which took place within 3 min after
20   application. Subsequently, an increase in leukocyte rolling
and adhesion was seen. No significant difference in leukocyte
accumulation was detected between the 20 and 30 minute
timepoints post 48/80 application, the mean of these two
timepoints was therefore used in the statistical analysis.
25   The venule-to venule variability in the model was quite high,
with baseline interaction ranging from 0 to 4.8 leukocytes
present in individual venules, and mast cell-induced
interaction ranging from 2.8 to 25.6 leukocytes present in the
PBS-treated group (coefficient of variability, CV, 37%).
30   Animal means showed much lower variability (CV, 16%); hence,
each individual venule was treated as a separate experimental
entity for statistical purposes.

        As seen in Fig. 29, intravenous treatment with mu
MAb PB1.3 inhibited the mast cell-induced intravascular
35   leukocyte accumulation by 90% when compared with the
formulation buffer control group. The non-reactive antibody
P6H6 did not have any effect in this model.

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Thus, administration of the anti-P-selectin
antibody, mu MAb Pb1.3, to rats completely prevents the
interaction of leukocytes with the vascular endothelium and
their subsequent migration into the tissues induced by
5   application of the mast cell degranulating agent 48/80.

Example 18
Mapping the Epitope of Mu MAb PB1.3
P-selectin is an integral membrane glycoprotein
10   found in secretory granules of platelets and endothelial
cells. After cellular activation, P-selectin is rapidly
redistributed to the plasma membrane. The mature molecule is
a protein with multiple domains, including a "lectin" region,
"EGF" region, nine tandem consensus "C3b-C4b Regulatory
15   Protein" (CRP) repeats, a transmembrane region, and a
cytoplasmic domain. See Johnston et al., *Cell*, 56:1033-1044
(1989) (herein incorporated by reference in its entirety for
all purposes) for the cloning and description of P-selectin.
Fig. 30 shows a proposed folding pattern of the domains of P-
20   selectin.

P-Selectin Deletion Analyses
In order to map the epitope specifically bound by mu
MAb PB1.3, mutants of recombinant P-selectin were constructed
25   by deleting consecutive CRP domains (Fig. 31). This site-
specific mutagenesis was accomplished by PCR amplification
using primers specific to each CRP domain (Table 6). The
template used for PCR-mutagenesis was a pUC DNA clone of P-
selectin which was truncated and mutated at DNA sequence
30   position 2354 (sequence positions are in accordance with the
published sequence of P-selectin of Johnston et al., *supra*.
This template plasmid pGΔ1,T which was derived initially by *in
vitro* mutagenesis, lacks the trans-membrane domain and the
cytoplasmic carboxy-terminus of P-selectin, and encodes
35   instead the carboxy-terminal 11 amino acid residues of SV40
Large T antigen. This SV40-derived C-terminus is recognized by
the monoclonal antibody KT-3. (*See* MacArthur et al., *Journal
of Virology* 52:483-491 (1984.)) After PCR amplification, the

deleted DNA molecules were purified, ligated, transformed into
bacteria, cloned and sequenced as described below. DNAs with
the appropriate linkage of CRP to the T-antigen tag sequence
were subcloned into the pcDNA1 expression vector (Invitrogen)
5   and transfected into COS cells.

          PCR mutagenesis was performed on plasmid pGMPΔ1,T.
After phosphorylation at the 5'-end, the primers in Table 6
were added to individual PCR reactions at 1 μM, together with
the T-antigen sequence primer lys-1. The PCR was performed in
10   a Perkin Elmer-Cetus machine for 30 cycles at 95°C for 50 sec,
50°C for 1 min. and 73°C for 4 min. using Pfu polymerase
(Stratagene) according to the manufacturer's recommended
conditions. After purification by agarose gel electrophoresis
and elution by Qiaex (Qiagen Corp.), the mutated pUC DNAs were
15   ligated and transformed into *E. coli* InvF'α cells
(Invitrogen). Cloned DNAs carrying the desired mutation were
identified by DNA sequence analysis and the SalI-HpaI
fragments containing the mutated P-selectin gene cloned into
the XhoI and XbaI sites of the expression vector, pcDNAI
20   (Invitrogen). The pcDNA clones were transformed into *E. coli*
strain MC1061 (Invitrogen) and purified plasmid DNAs used for
transfection into COS.

          DEAE-Dextran Transfection Method
          This method is described in Kriegler, M., *Gene*
25   *Transfer and Expression: A Laboratory Manual*, W.H. Freeman and
Company (1990). COS cells were seeded at 1 x 10^6 cells/100 mm
dish in DMEM (Biowhittaker), 10% fetal bovine serum (FBS). On
day two, plasmid DNA was ethanol precipitated, and resuspended
at a concentration of 20 μg/mL in sterile TE (10 mM Tris, pH
30   8.0, 1 mM EDTA). 150 μL of DNA was mixed with 300 μl of
sterile TBS (Tris Buffered Saline, 140 mM NaCl, 5 mM KCl, 1.4
mM Na_2HPO_4, 25 mM Tris-base, pH 7.5, 1.0 mM $CaCl_2$, and 0.5 mM
$MgCl_2$) and with 300 μl of sterile DEAE dextran (Sigma, #D-
9885, 1 mg/ml in TBS). The growth media was aspirated, and
35   the cell monolayers were washed once with PBS, and once with
TBS. 750 μl of the DNA/DEAE dextran/TBS mixture was added to
the monolayer. The dish was incubated at ambient temperature
inside a laminar flood hood rocking the dish every 5 min for

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1 h. After the 1 h incubation, the DNA solution was aspirated
and the cells were washed once with TBS and then once with
PBS. The cells were incubated in a complete medium
supplemented with 100 μM chloroquine (Sigma, #C-6628), 37°C,
5 5% CO_2. After 4 h, the medium was replaced with complete
media, and the cells were incubated at 37°C and 5% CO_2. After
48 h post-transfection, the cells were fed with DMEM growth
medium lacking serum. 24 h later the medium was harvested,
the cell debris removed by centrifugation at 1500 rpm for
10 5 min in a tabletop clinical centrifuge.

<u>Capture ELISA to Analyze Secreted P-Selectin Mutant</u>

<u>Proteins</u>

A 96-well Costar plate was coated with KT-3 antibody
(produced in ascites and purified by Protein G Sepharose,
15 Pharmacia) 100 μl/well at 40 μg/ml at 4°C overnight. The
plate was washed 3x with DPBS, blocked for 1 h at ambient
temperature with 250 μl/well of DPBS + 1% BSA. The plate was
washed 3x with DPBS, and incubated with COS cell supernatants
100 μl/well for 2 h at ambient temperature. After washing the
20 plate 3x with DPBS the bound P-selectin was detected by adding
either 100 μl/well of (a) 1 ng/ml rabbit anti-P-selectin
polyclonal Ig, or (b) biotinylated mu MAb PB1.3, or (c)
biotinylated P6H6 diluted in DPBS/1% BSA for 1 h at ambient
temperature. After washing the plate 3x with DPBS the rabbit
25 antibody was detected using 100 μl/well of a 1:1000 dilution
of HRP-conjugated goat anti-rabbit antibody (Biorad), blocked
with tissue culture media from the KT-3 cell line (10%) in
DPBS/1% BSA for 30 min at ambient temperature. The
biotinylated antibodies were detected using 100 μl/well of a
30 1:1000 dilution of HRP-streptavidin (Pierce) for 30 min at
ambient temperature. The bound HRP was incubated with TMB and
quenched with 1 M phosphoric acid. Figure 32 shows the signal
less the background from supernatants of mock transfected COS
cells. The "Mock" column is the supernatant from cells
35 transfected with a full length form of P-selectin, which lacks
the SV40 T-antigen tag, and therefore is not detected in this
KT-3 capture ELISA. The X-axis specifies the number of CRPs
expressed by the various mutant P-selectin cDNAs, from all 9

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CRPs in column 2 to one CRP in column 10. The results show that, although P-selectin mutants lacking the carboxy-terminal 5 CRPs can be detected by the polyclonal rabbit Ig, these molecules do not react with PB1.3. This suggests that the

5   binding site for PB1.3 lies within the fifth CRP repeat domain.

In order to confirm the results from epitope mapping, the deletion analysis was extended to delete only the fifth CRP domain, leaving the carboxy-terminal sequences of P-

10   selectin intact (see Fig. 30). PCR amplimers were designed to remove the region of P-selectin encoding amino acids 407 to 468 (Table 6) using the same protocols described above. Cell supernatants were analyzed directly by ELISA with antibodies Mu MAb PB1.3 and P6H6 (Fig. 33). Although mu MAb PB1.3 binds

15   the intact P-selectin, it fails to bind the deleted molecule. P6H6 binds both molecules equivalently.

Synthetic Peptide Analyses

The mu MAb PB1.3 epitope was further characterized by synthesizing peptides spanning the 62 amino acid region of

20   the fifth CRP of P-selectin (Fig. 34) and screening for mu MAb PB1.3 binding by ELISA. The fifth CRP extends from amino acid sequence 407 to 468 according to the nomenclature of Johnston, et al. The peptide sequences are 968.01, a.a. # 408-426; 968.02, a.a. #418-436; 968.04, a.a. #408-433; 968.05,

25   an oxidized version of 968.04; 968.06, a.a. #428-447; and 968.08, a.a. #448-467. Each peptide was dissolved to 2mg/ml in dimethyl sulfoxide (DMSO) and coated onto a 96-well microtiter plated by dilution of 1 microgram into 100 μl PBS and dried at 37°C overnight. Coated wells were washed with

30   PBS and blocked with 250 μl/well of a solution of PBS/1% BSA for 30 min at ambient temperature. The wells were washed with PBS and incubated with 100μl/well of PB1.3 diluted to 1μg/ml in PBS/1%BSA for 1 h at room temperature. Bound mu MAb PB1.3 was detected with HRP-conjugated goat anti-mouse antibody

35   reagent and TMB substrate as described before. Fig. 34 shows a comparison PB1.3 reactivity with the peptides. The carboxy-terminal peptide, number 968.08 reacted strongly with PB1.3.

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Therefore the epitope recognized by PB1.3 maps to amino acids
448 to 467 in the fifth CRP of human P-selectin.

  For the purposes of clarity and understanding, the
invention has been described in these examples and the above
5 disclosure in some detail. It will be apparent, however, that
certain changes and modifications may be practiced within the
scope of the appended claims. All publications and patent
applications cited above are hereby incorporated by reference
in their entirety for all purposes to the same extent as if
10 each were so individually denoted.

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TABLE 1 (1 of 4)

ALIGNMENT OF AMINO ACID SEQUENCES LEADING TO THE DESIGN OF RESHAPED HUMAN PB1.3 HEAVY CHAIN VARIABLE REGION

Kabat	#	FR or CDR	mouse 1747	mouse II A	human I	human 21/28'CL	RH V$_a$ 1747	Comment
1	1	Fk1	E	E	Q	PCA	E	close to H1, possible role in antigen binding
2	2		A	V	V	V	A	unusual AA. close to H1, possible role in antigen binding
3	3		Q	Q	Q	Q	Q	
4	4		L	L	L	L	L	
5	5		Q	Q	V	V	V	
6	6		Q	Q	Q	Q	Q	
7	7		S	S	S	S	S	
8	8		G	G	G	G	G	
9	9		P	P	A	A	A	
10	10		E	E	E	E	E	
11	11		L	L	V	V	V	
12	12		V	V	K	K	K	
13	13		E	K	K	K	K	
14	14		P	P	P	P	P	
15	15		G	G	G	G	G	
16	16		A	A	A	A	A	
17	17		S	S	S	S	S	
18	18		V	V	V	V	V	
19	19		K	K	K	K	K	
20	20		V	I	V	V	V	
21	21		S	S	S	S	S	
22	22		C	C	C	C	C	
23	23		K	K	K	K	K	
24	24		A	A	A	A	A	
25	25		S	S	S	S	S	
26	26		G	G	G	G	G*	
27	27		Y	Y	Y	Y	Y*	
28	28		T	T	T	T	T~	
29	29		F	F	F	F	F*	

TABLE 1 (2 of 4)

30	30	FR1	T	T	T	T	T•	
31	31	CDR1	N	D	S	S	N•	
32	32	:	Y	Y	Y	Y	Y•	
33	33	:	V	Y	A	A	V	
34	34	:	M	M	I	M	M•	
35	35	:	H	N	S	H	H	
35A		:	-	-	-	-	-	
35B		CDR1	-	-	-	-	-	
36	36	FR2	W	W	W	W	W	
37	37	:	V	V	V	V	V	
38	38	:	K	K	R	R	R	
39	39	:	Q	Q	Q	Q	Q	
40	40	:	K	S	A	A	A	
41	41	:	P	P	P	P	P	
42	42	:	G	G	G	G	G	
43	43	:	Q	K	Q	Q	Q	
44	44	:	G	S	G	R	R	
45	45	:	L	L	L	L	L	
46	46	:	E	E	E	E	E	
47	47	:	W	W	W	W	W	
48	48	:	I	I	M	M	M	
49	49	FR2	G	G	G	G	G	
50	50	CDR2	F	D	W	W	F	
51	51	:	I	I	I	I	I	
52	52	:	N	N	N	N	N	
52A	53	:	P	P	P	A	P•	
52B		:	-	-	Y	-	-	
52C		:	-	-	-	-	-	
53	54	:	S	G	G	G	S•	
54	55	:	N	N	N	N	N•	
55	56	:	D	G	G	G	D•	
56	57	:	G	G	D	N	G	
57	58	:	P	T	T	T	P	
58	59	:	K	S	N	K	K	
59	60	:	Y	Y	Y	T	Y	
60	61		N	N	A	S	N	
61	62		E	O	O	O	E	
62	63		R	K	K	K	R	

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TABLE 1 (3 of 4)

63	64	:	F	F	F	F	F	
64	65	'	K	K	Q	Q	K	
65	66	CDR2	N	G	G	G	N	
66	67	FR3	K	K	R	R	R	
67	68	!	A	A	V	V	V	
68	69	!	T	T	T	T	T	
69	70	!	L	L	I	I	I	
70	71	!	T	T	T	T	T	
71	72	!	S	V	A	R	S*	H2 canonical structure, may be supporing H2
72	73	'	D	D	D	D	D	
73	74	.	K	K	T	T	T	
74	75	!	S	S	S	S	S	
75	76	'	S	S	T	A	A	
76	77	!	S	S	S	S	S	
77	78	!	T	T	T	T	T	
78	79	!	A	A	A	A	A	
79	80	'	Y	Y	Y	Y	Y	
80	81	.	M	M	M	M	M	
81	82	'	E	Q	E	E	E	
82	83	.	L	L	L	L	L	
82A	84	:	S	S	S	S	S	
82B	85	:	S	S	S	S	S	
82C	86	!	L	L	L	L	L	
83	87	:	T	T	R	R	R	
84	88	:	S	S	S	S	S	
85	89	.	E	E	E	E	E	
86	90	'	V	D	D	D	D	
87	91	:	S	S	T	T	T	
88	92	.	A	A	A	A	A	
89	93	'	V	V	V	V	V	
90	94	:	Y	Y	Y	Y	Y	
91	95	.	F	Y	Y	Y	Y	
92	96		C	C	C	C	C	
93	97	:	A	A	A	A	A	
94	98	FR3	R	R	R	R	R*	
95	99	CDR3	A	G	A	G	A	

TABLE 1 (4 of 4)

96	:00	:	R	X	P	.	R	
97	101	:	P	Y	G	.	P	
98	102	:	G	Y	Y	.	G	
99	103	:	F	S	G	G	F	
100	104	:	D	S	S	.	D	
100A		:	.	S	G	.	.	
100B		.	.	Y	G	.	.	
100C		.	.	M	G	.	.	
100D		:	.	X	C	.	.	
100E		.	.	A	Y	.	.	
100F		.	.	X	R	Y	.	
100G		:	.	X	G	Y	.	
100H		.	.	Y	D	G	.	
100I	105	:	W	Y	Y	S	W	
100J	106	:	Y	A	.	G	Y	
100K	107	:	F	F	F	S	F	
101	108	:	D	D	D	N	D	
102	109	CDR3	V	Y	Y	Y	V	
103	:10	FR4	W	W	W	W	W	
104	111		G	G	G	G	G	
105	112	.	A	Q	Q	Q	Q	
106	113	:	G	G	G	G	G	
107	114	.	T	T	T	T	T	
108	115	:	T	T	L	L	L	
109	116	.	V	V	V	V	V	
110	117		T	T	T	T	T	
111	118	.	V	V	V	V	V	
112	119	.	S	S	S	S	S	
:13	:20	FR4	S	S	S	.	S	

TABLE 2 (1 of 4)

ALIGNMENT OF AMINO ACID SEQUENCES LEADING TO THE DESIGN OF RESHAPED PB1.3 LIGHT CHAIN VARIABLE REGION

Kabat	#	FR or CDR	mouse 1747	mouse κ-V	human κ-l	human DEN	RH V$_L$ 1747	Comment
1	1	FR1	D	D	D	D	D	
2	2		I	I	I	I	I*	
3	3		V	Q	Q	Q	Q	
4	4		M	M	M	M	M	
5	5		T	T	T	T	T	
6	6		Q	Q	Q	Q	Q	
7	7		S	S	S	S	S	
8	8		Q	P	P	P	P	
9	9		K	S	S	S	S	
10	10		F	S	S	T	T	
11	11		M	L	L	L	L	
12	12		S	S	S	S	S	
13	13		T	A	A	A	A	
14	14		S	S	S	S	S	
15	15		V	L	V	V	V	
16	16		G	G	G	G	G	
17	17		D	D	D	D	D	
18	18		R	R	R	R	R	
19	19		V	V	V	V	V	
20	20		S	T	T	T	T	
21	21		V	I	I	I	_V_	supporting L1. I may disrupt the core
22	22		T	T	T	T	T	
23	23	FR1	C	C	C	C	C	
24	24	CDR1	K	R	R	R	K	
25	25		A	A	A	T	A*	
26	26		S	S	S	S	S*	
27	27		Q	Q	Q	Q	Q*	
27A	28		N	D	S	S	N	
27B			.	.	L	.	.	
27C			.	.	V	.	.	
27D			.	.	.	.	.	
27E			.	.	.	.	.	

TABLE 2 (2 of 4)

27F		:	.	.	.	.	.	
28	29	:	V	D	S	I	V•	
29	30	:	A	I	I	S	A•	
30	31	:	T	S	S	R	T•	
31	32	:	N	N	N/S	W	N•	
32	33	:	V	Y	Y	L	V•	
33	34			V	L	L	A	V•	
34		CDR1	-	N	A	.	.	
35	35	FR2	W	W	W	W	W	
36	36	:	Y	Y	Y	Y	Y	
37	37	:	Q	Q	Q	Q	Q	
38	38	:	Q	Q	Q	Q	Q	
39	39	:	R	K	K	K	K	
40	40	:	P	P	P	P	P	
41	41	:	G	G	G	G	G	
42	42	:	Q	Q	K	E	E	
43	43	:	S	S	A	A	A	
44	44	:	P	P	P	P	P	
45	45	:	K	K	K	K	K	
46	46	.	A	L	L	L	A̲	may be supporting L2. L may alter the orientation of L2
47	47	:	L	L	L	L	L	
48	48	:	I	I	I	I	I•	
49	49	FR2	Y	Y	Y	Y	Y	
50	50	CDR2	T	Y	A	G	T•	
51	51	:	A	A	A	A	A•	
52	52	:	S	S	S	S	S•	
53	53	:	Y	R	S	N	Y	
54	54	:	R	L	L	L	R	
55	55	:	F	H	E	E	F	
56	56	CDR2	S	S	S	S	S	
57	57	FR3	G	G	G	G	G	
58	58	:	V	V	V	V	V	
59	59		P	P	P	P	P	

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TABLE 2 (3 of 4)

60	60	!	E	S	S	S	E̲	close to residue 54 in L2, on the surface and may bind Ag
61	61	!	R	R	R	R	R	
62	62	!	F	F	F	F	F	
63	63	!	S	S	S	S	S	
64	64	!	G	G	G	G	G*	
65	65	!	S	S	S	S	S	
66	66	!	G	G	G	G	G	
67	67	!	S	S	S	S	S	
68	68	'	G	G	G	G	G	
69	69	!	T	T	T	T	T	
70	70	!	D	D	D	E	D̲	may have charge interaction with residue 24 in L1.
71	71	!	F	Y	F	F	F*	
72	72	!	T	S	T	T	T	
73	73	!	L	L	L	L	L	
74	74	!	T	T	T	T	T	
75	75	!	I	I	I	I	I	
76	76	!	T	S	S	S	S	
77	77	!	N	N	S	S	S	
78	78	!	V	L	L	L	L	
79	79	!	Q	E	Q	Q	Q	
80	80	!	S	Q	P	S	S	
81	81	!	E	E	E	D	D	
82	82	!	D	D	D	D	D	
83	83	!	L	I	F	F	F	
84	84	!	A	A	A	A	A	
85	85	!	D	T	T	T	T	
86	86	!	Y	Y	Y	Y	Y	
87	87	'	F	F	Y	Y	Y	
88	88	FR3	C	C	C	C	C	
89	89	CDR3	Q	Q	Q	Q	Q	
90	90	!	Q	Q	Q	Q	Q*	
91	91	!	Y	G	Y	Y	Y*	
92	92	!	N	N	N	D	N*	
93	93	!	N	T	S	S	N*	

9 1

TABLE 2 (4 of 4)

94	94	I	Y	L	L	F	Y*	
95	95	I	P	P	P	P	P*	
95A		I	.	.	E	.	.	
95B		I	.	.	.	.	.	
95C		I	.	.	.	.	.	
95D		I	.	.	.	.	.	
95E		I	.	.	.	.	.	
95F		I	.	.	I	.	.	
96	96	I	Y	R	W	Y	Y*	
97	97	CDR3	T	T	T	T	T	
98	98	FR4	F	F	F	F	F	
99	99	I	G	G	G	G	G	
100	100	I	G	G	Q	Q	Q	
101	101	I	G	G	G	G	G	
102	102	I	T	T	T	T	T	
103	103	I	K	K	K	K	K	
104	104	I	V	L	V	L	L	
105	105	I	E	E	E	E	E	
106	106	I	I	I	I	I	I	
106A		I	.	.	.	.	.	
107	107	FR4	Q	K	K	K	K	

9 2

TABLE 3

DIFFERENCES IN AMINO ACID SEQUENCES FOR THE
HEAVY CHAIN VARIABLE DOMAINS FOR THE
THREE RESHAPED VERSIONS OF PB1.3

CONSTRUCT   AMINO ACID

	1	2	73
1748RH$_A$	GLU	ALA	THR
1748RH$_B$	GLN	VAL	THR
1748RH$_C$	GLU	ALA	LYS

93

TABLE 4

DIFFERENCES IN AMINO ACID SEQUENCES FOR THE
LIGHT CHAIN VARIABLE DOMAINS FOR THE
FOUR RESHAPED VERSIONS OF PB1.3

CONSTRUCT   **AMINO ACID**

	60	70
1748RL$_A$	GLU	ASP
1748RL$_B$	SER	ASP
1748RL$_C$	GLU	GLU
1748RL$_D$	SER	GLU

94

TABLE 5

ANTIBODY PRODUCTION RATES FOR AMPLIFIED CHO CELL LINES
PRODUCING THREE DIFFERENT VERSIONS OF HUMANIZED PB1.3 (CY1748)
(micrograms/10^6 cells/day)

Antibody	Unamplified	1st round	2nd round	3rd round
1748A-IgG1	0.36	2.1	8.2	29.6
	0.36	2.1	13.7	21.8
	0.36	0.90	9.9	19.7
1748B-IgG1	0.10	1.6	23.9	
	0.24	1.5	21.1	
	< 0.2	7.3	18.6	
	0.10	1.6	17.8	
1748B-IgG4	< 0.3	< 2.0	33.0	
	< 0.3	< 2.0	26.9	
	0.75	2.6	23.2	
	< 0.3	3.0	20.8	
	0.80	6.5	20.8	
	< 0.3	< 2.0	19.4	
	0.78	2.5	18.6	
	0.78	8.8	17.8	

95

TABLE 6

DNA SEQUENCE OF THE PCR AMPLIMERS USED TO GENERATE HUMAN P-SELECTIN CRP DELETION MUTANTS

PCR 3'-amplimer to remove CRP Number:		Predicted amino acid sequence of mutant		
		CRP		TAg
9	5'-TTTCACAGCTCTGCATGC-3'	...CRAVK	TIQEA	LK...
8 - 9	5'-TGCTATGCCTTTGCAGGT-3'	...CKGIA	TIQEA	LK...
7 - 9	5'-CTTGATGGCTTCACACAT-3'	...CEAIK	TIQEA	LK...
6 - 9	5'-GGGAATGGCTTGGCATTC-3'	...CQAIP	TIQEA	LK...
5 - 9	5'-CTGCAAAGCTTGACAGAC-3'	...CQALQ	TIQEA	LK...
4 - 9	5'-CGAAATAGCCTCACAGGT-3'	...CEAIS	TIQEA	LK...
3 - 9	5'-CTGCACAGCTTTACACAC-3'	...CKAVQ	TIQEA	LK...
2 - 9	5'-CTGGGCAGCTAAACACTG-3'	...CLAAQ	TIQEA	LK...

5'-amplimer, lys-1

    5'-AAACCTCCCACACCCCT-3'

To Delete only CRP#5   these two amplimers were used:

5'-amplimer   5'-TGCACACCTTTGCTAAGC-3'
3'-amplimer   5'-CTGCAAAGCTTGACAGAC-3'

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<u>WHAT IS CLAIMED IS:</u>

1   1.   A blocking P-selectin antibody that
2   competitively inhibits the binding of an antibody secreted by
3   a cell line designated ATCC Accession No. HB11041 to P-
4   selectin as measured by a competitive inhibition assay.

1   2.   The blocking P-selectin antibody of claim 1
2   that inhibits neutrophil binding to P-selectin in the absence
3   of Ca^{++}.

1   3.   The blocking P-selectin antibody of claim 1
2   that inhibits neutrophil binding to P-selectin in the presence
3   of a peptide CQNRYTDLVAIQNKNE.

1   4.   The blocking P-selectin antibody of claim 1
2   that is an IgG immunoglobulin.

1   5.   The blocking P-selectin antibody of claim 1
2   that is a murine antibody.

1   6.   The blocking P-selectin antibody of claim 1
2   that is produced by a cell line designated ATCC Accession No.
3   HB11041.

1   7.   The antibody of claim 1 that is a Fab, Fab'
2   $F(ab')_2$, Fabc or Fv fragment.

1   8.   A method for detecting P-selectin, the method
2   comprising:
3   administering the antibody of claim 1 to a patient
4   or a tissue sample therefrom; and
5   detecting complexes formed by specific binding
6   between the antibody and P-selectin present in the target
7   sample.

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1   9.   A method for treating a disease of the immune
2 system, the method comprising administering to a patient
3 having the disease a therapeutically effective dose of a
4 pharmaceutical composition comprising a pharmaceutically
5 acceptable carrier and the blocking P-selectin antibody of
6 claim 1.

1   10.   The method of claim 9, wherein the blocking P-
2 selectin antibody is IgG_1.

1   11.   The method of claim 9, wherein the blocking P-
2 selectin antibody is murine.

1   12.   The method of claim 9, wherein the blocking P-
2 selectin antibody is produced by the cell line having ATCC
3 Accession No. HB11041.

1   13.   The method of claim 9, wherein the disease is a
2 result of acute lung injury.

1   14.   The method of claim 9, wherein the disease is
2 ischemia-reperfusion injury.

1   15.   The method of claim 9, wherein the blocking P-
2 selectin antibody is administered intravenously.

1   16.   A method for treating a condition of the immune
2 system, the method comprising administering to a patient
3 having the condition a therapeutically effective dose of a
4 pharmaceutical composition comprising a pharmaceutically
5 acceptable carrier and the blocking P-selectin antibody of
6 claim 1.

1   17.   A method of inhibiting adhesion of a leukocyte
2 to an endothelial cell or platelet, the method comprising
3 administering a therapeutically effective amount of a
4 pharmaceutical composition comprising the antibody of claim 1
5 and a pharmaceutically acceptable carrier.

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1   18.   A pharmaceutical composition comprising a
2   pharmaceutically acceptable carrier and the blocking P-
3   selectin antibody of claim 1.

1   19.   The pharmaceutical composition of claim 18,
2   wherein the blocking P-selectin antibody is secreted by the
3   cell line designated ATCC Accession No. HB11041.

1   20.   The pharmaceutical composition of claim 19,
2   wherein the antibody is murine.

1   21.   A composition comprising a cell line designated
2   ATCC Accession No. HB11041.

1   22.   A humanized immunoglobulin comprising a
2   humanized heavy chain and a humanized light chain:
3   (1) the humanized light chain comprising three
4   complementarity determining regions (CDR1, CDR2 and CDR3)
5   having amino acid sequences from the corresponding
6   complementarity determining regions of a mouse PB1.3
7   immunoglobulin light chain, and a variable region framework
8   from a human kappa light chain variable region framework
9   sequence; and
10   (2) the humanized heavy chain comprising three
11   complementarity determining regions (CDR1, CDR2 and CDR3)
12   having amino acid sequences from the corresponding
13   complementarity determining regions of a mouse PB1.3
14   immunoglobulin heavy chain, and a variable region framework
15   from a human heavy chain variable region framework sequence;
16   wherein the humanized immunoglobulin specifically
17   binds to P-selectin with a binding affinity having a lower
18   limit of about 10^7 M^{-1} and an upper limit of about five-times
19   the binding affinity of the mouse PB1.3 immunoglobulin.

1   23.   The humanized immunoglobulin of claim 22,
2   wherein:
3   the human kappa light chain variable region
4   framework sequence is substituted in at least one position

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5   selected from a first group consisting of L21, L46, L60 and
6   L70, by an amino acid present in the equivalent position of
7   the mouse PB1.3 immunoglobulin light chain variable region
8   framework sequence; and
9   the human heavy chain variable region framework
10   sequence is substituted in at least one position selected from
11   a second group consisting of H1, H2, H71 and H73, by an amino
12   acid present in the equivalent position of the mouse PB1.3
13   heavy chain variable region framework sequence.

1   24.   The humanized immunoglobulin of claim 23,
2   wherein the humanized heavy chain variable region framework is
3   a 21/28'CL heavy chain variable region framework sequence.

1   25.   The humanized immunoglobulin of claim 24,
2   wherein the humanized light chain variable region framework is
3   a DEN light chain variable region framework sequence.

1   26.   The humanized immunoglobulin of claim 25,
2   wherein the humanized light chain variable region framework is
3   substituted in at least two positions from the first group.

1   27.   The humanized immunoglobulin of claim 26,
2   wherein the humanized heavy chain variable region framework is
3   substituted in at least two positions from the second group.

1   28.   The humanized immunoglobulin of claim 22,
2   wherein the humanized light chain comprises the 1748RLA mature
3   light chain variable region amino acid sequence shown in
4   Figure 14 (SEQ. ID. No. ___).

1   29.   The humanized immunoglobulin of claim 22,
2   wherein the humanized light chain comprises the 1748RLB mature
3   light chain variable region amino acid sequence shown in
4   Figure 15 (SEQ. ID. No. ___).

1   30.   The humanized immunoglobulin of claim 22,
2   wherein the humanized light chain comprises the 1748RLC mature
3   light chain variable region amino acid sequence shown in
4   Figure 16 (SEQ. ID. No. ___).

1   31.   The humanized immunoglobulin of claim 22,
2   wherein the humanized light chain comprises the 1748RLD mature
3   light chain variable region amino acid sequence shown in
4   Figure 17 (SEQ. ID. No. ___).

1   32.   The humanized immunoglobulin of claim 22,
2   wherein the humanized heavy chain comprises the 1748RHA mature
3   heavy chain variable region amino acid sequence shown in
4   Figure 11 (SEQ. ID. No. ___).

1   33.   The humanized immunoglobulin of claim 22,
2   wherein the humanized heavy chain comprises the 1748RHB heavy
3   chain variable region amino acid sequence shown in Figure 12
4   (SEQ. ID. No. ___).

1   34.   The humanized immunoglobulin of claim 22,
2   wherein the humanized heavy chain comprises the 1748RHC mature
3   heavy chain variable region amino acid sequence shown in
4   Figure 13 (SEQ. ID. No. ___).

1   35.   The humanized immunoglobulin of claim 31,
2   wherein the humanized heavy chain comprises the 1748RHB mature
3   heavy chain variable region amino acid sequence shown in
4   Figure 12 (SEQ. ID. No. _____).

1   36.   The humanized immunoglobulin of claim 22 that
2   is a Fab, Fab', F(ab')$_2$, Fabc or Fv fragment.

1   37.   The humanized immunoglobulin of claim 35
2   further comprising a constant region domain.

1   38.   The humanized immunoglobulin of claim 37,
2   wherein the constant region domain has an effector function.

1   39.   The humanized immunoglobulin of claim 35
2   wherein the constant region domain lacks an effector function.

1   40.   The humanized immunoglobulin of claim 38,
2   wherein the effector function is capable of complement
3   fixation or antibody dependent cellular toxicity.

1   41.   A nucleic acid encoding the heavy chain of the
2   humanized immunoglobulin of claim 22.

1   42.   A nucleic acid encoding the light chain of the
2   humanized immunoglobulin of claim 22.

1   43.   A computer programmed to display a three-
2   dimensional representation of the humanized immunoglobulin of
3   claim 22 on a monitor or a printer.

1   44.   A pharmaceutical composition comprising the
2   humanized antibody of claim 35, and a pharmaceutically
3   acceptable carrier therefor.

1   45.   A pharmaceutical composition comprising the
2   humanized immunoglobulin of claim 22 and a pharmaceutically
3   acceptable carrier therefor.

1   46.   The pharmaceutical composition of claim 45,
2   wherein the humanized immunoglobulin is a Fab fragment.

1   47.   A method for detecting P-selectin, the method
2   comprising:
3   administering the humanized immunoglobulin of
4   claim 35 to a patient or a tissue sample therefrom; and
5   detecting complexes formed by specific binding
6   between the immunoglobulin and P-selectin present in the
7   target sample.

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1   48.   A method of inhibiting adhesion of a leukocyte
2   to an endothelial cell or platelet, the method comprising
3   administering a therapeutically effective amount of the
4   pharmaceutical composition of claim 45.

1   49.   A method of treating a patient suffering from a
2   disease of the immune system, the method comprising
3   administering to the patient having the disease a
4   therapeutically effective amount of the pharmaceutical
5   composition of claim 45.

1   50.   The method of claim 49, wherein the disease is
2   a result of acute lung injury.

1   51.   The method of claim 49, wherein the disease is
2   ischemia-reperfusion injury.

1   52.   The method of claim 51, wherein the patient is
2   suffering from epidermal, myocardial, renal, cerebral,
3   splenic, hepatic, spinal, splanchnic, pulmonary, partial-body,
4   or whole-body ischemia.

1   53.   A method of treating a patient suffering from a
2   condition of the immune system, the method comprising
3   administering to the patient having the condition a
4   therapeutically effective amount of the pharmaceutical
5   composition of claim 45.

1   54.   A stable cell line comprising:
2   a nucleic acid segment encoding the heavy chain of
3   the humanized immunoglobulin of claim 35, the segment operably
4   linked to a promoter to allow expression of the heavy chain;
5   a second nucleic acid segment encoding the light
6   chain of the humanized immunoglobulin of claim 35, the second
7   segment operably linked to a second promoter to allow
8   expression of the light chain;
9   wherein the stable cell line is capable of producing
10   the humanized immunoglobulin of claim 35.

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1   55.   The cell line of claim 54 that is capable of
2   producing about 30 µg of the humanized immunoglobulin/10^6
3   cells/day.

1   56.   The cell line of claim 55 designated CHO-48B4,
2   deposited as ATCC _____.

1   57.   A purified P-selectin polypeptide having up to
2   100 amino acids, the up to 100 amino acids comprising at least
3   five contiguous amino acids from between positions 448 and 467
4   of the amino sequence shown in Fig. 34 (SEQ. ID. No. ____).

1   58.   The purified polypeptide of claim 57, wherein
2   the up to 100 amino acids comprise the contiguous segment of
3   amino acids between positions 448 and 467 of the amino acid
4   sequence shown in Fig. 34 (SEQ. ID. No. ____).

1   59.   The purified polypeptide of claim 58 consisting
2   essentially of the contiguous segment of amino acids between
3   positions 448 and 467 of the amino acid sequence shown in
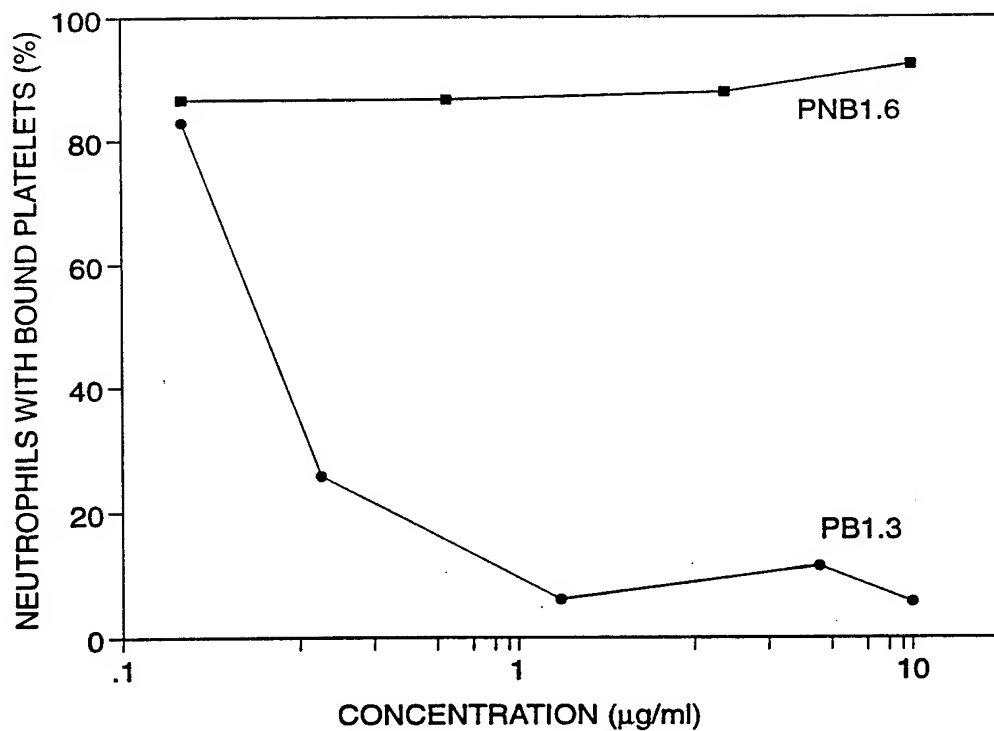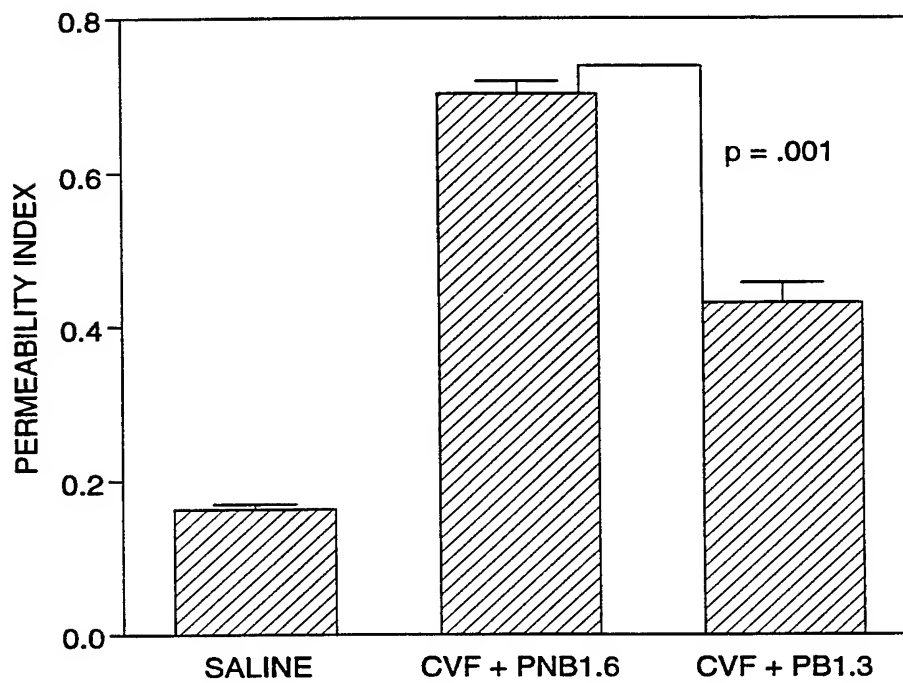4   Fig. 34 (SEQ. ID. No. ____).

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FIG. 1

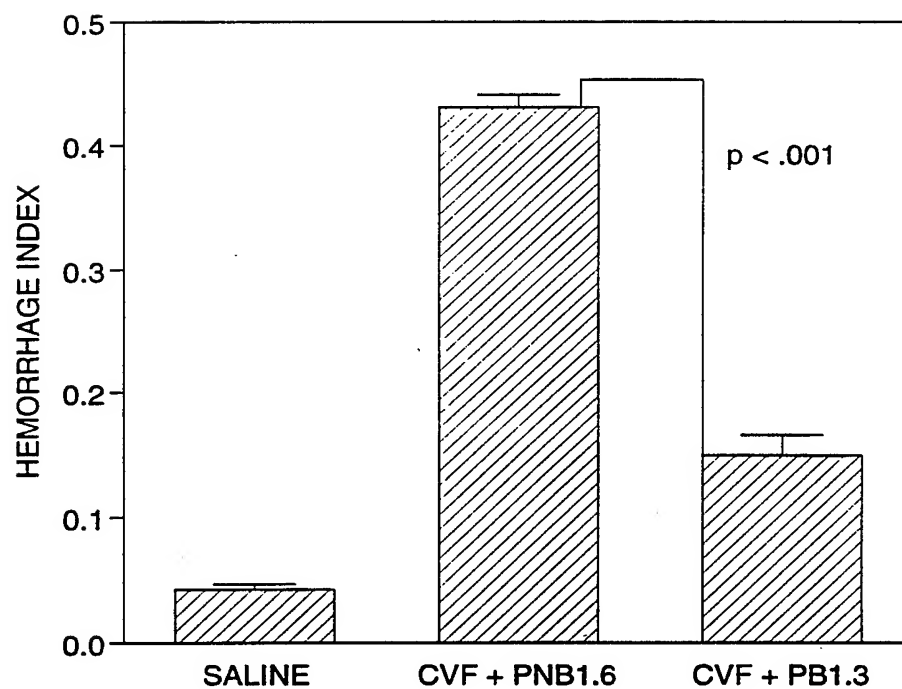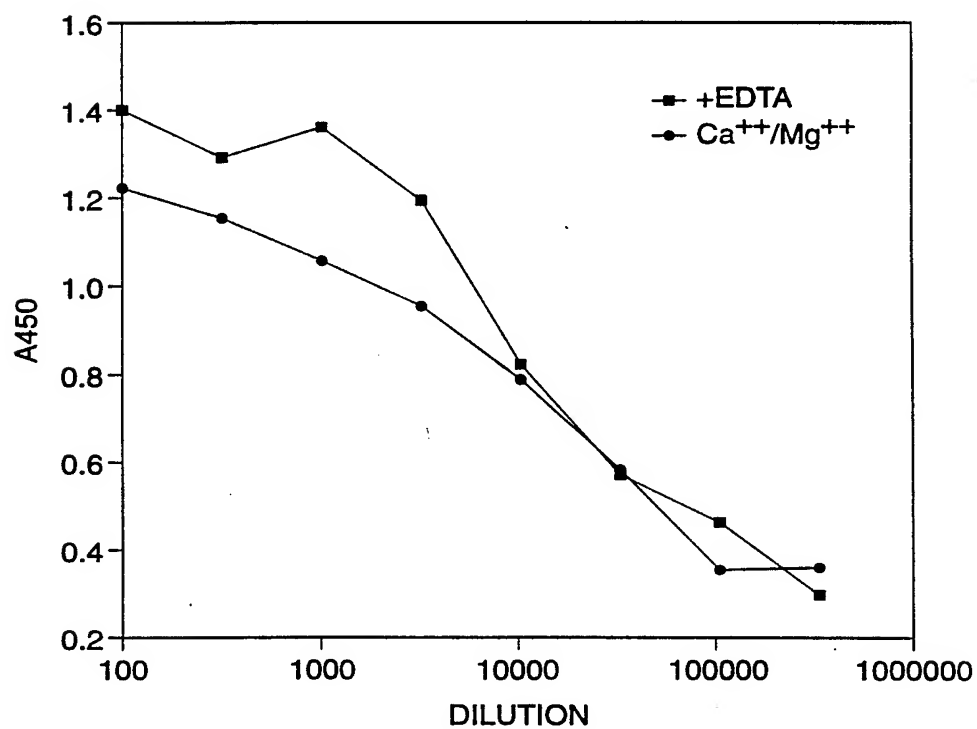*FIG. 2A*
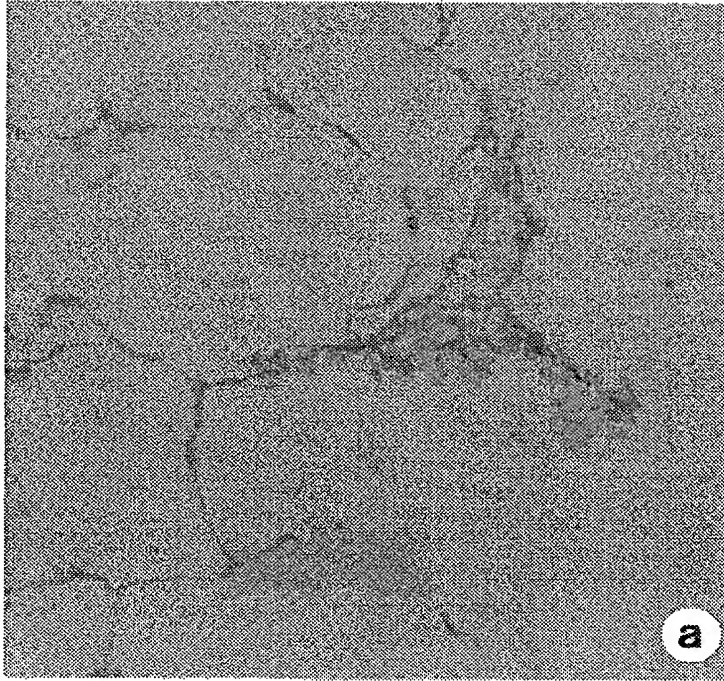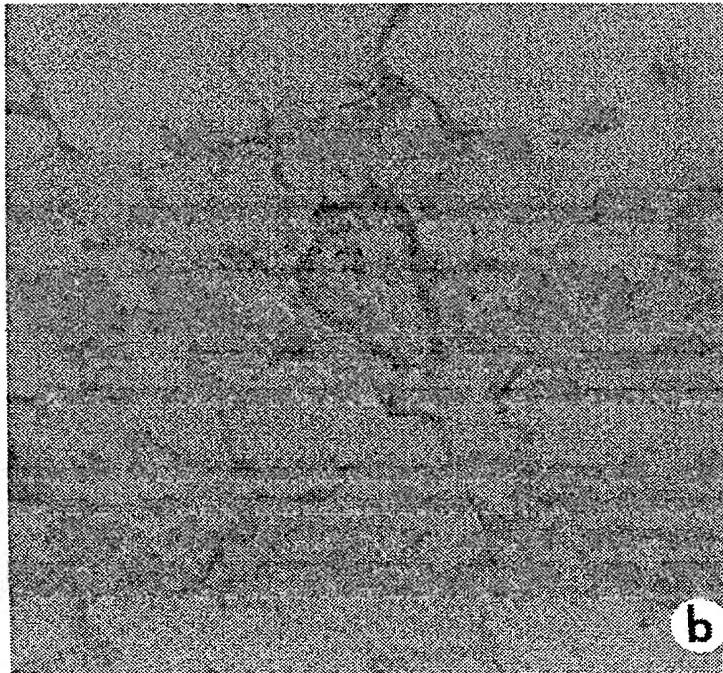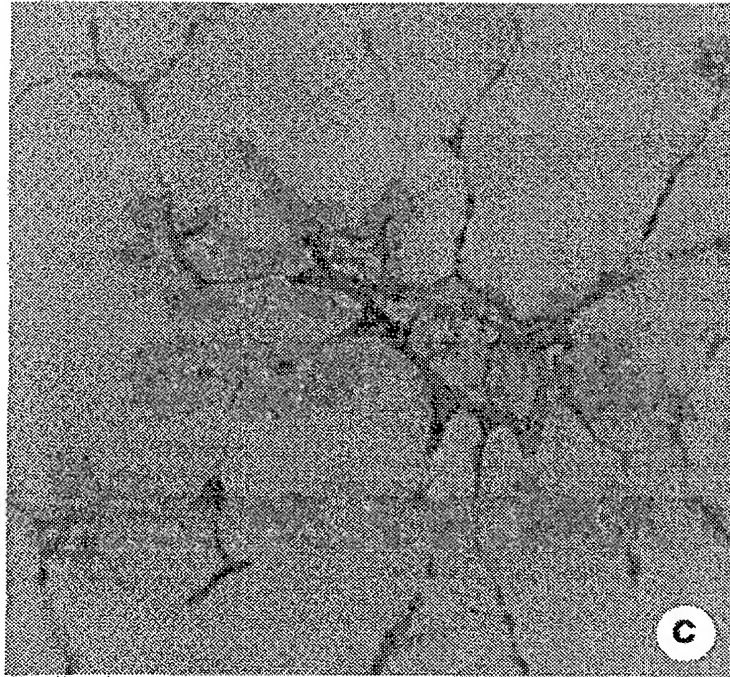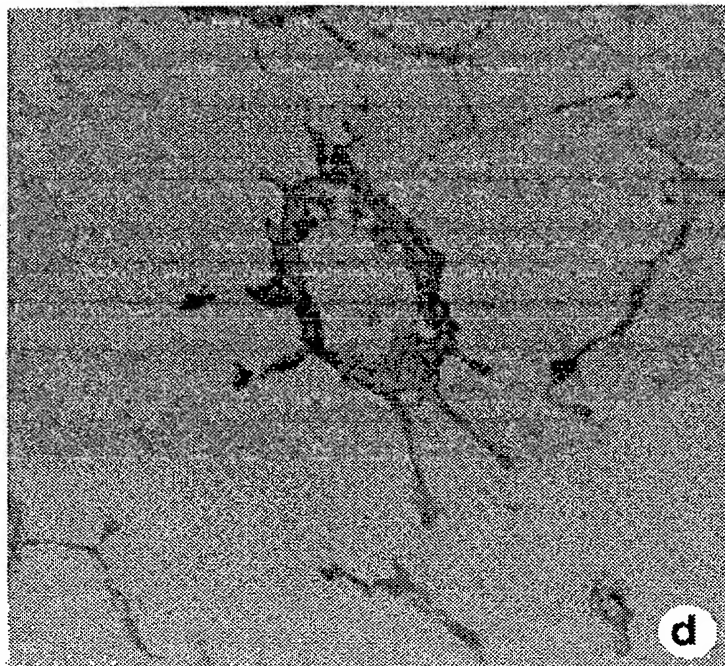
FIG. 2B

FIG. 4

FIG. 3A.

FIG. 3B.

FIG. 3C.

FIG. 3D.

FIG. 3E.
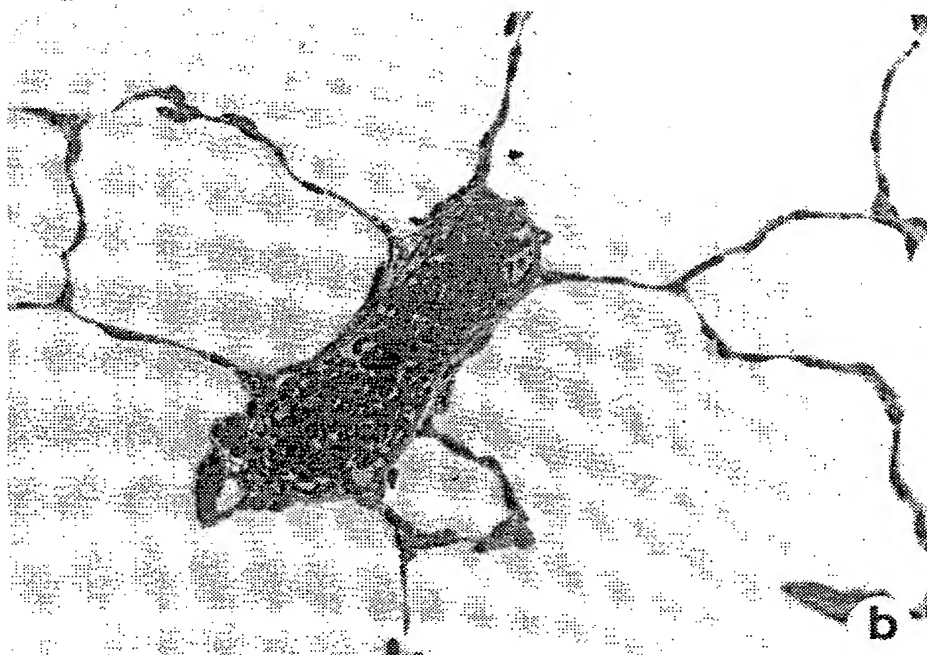
FIG. 3F.

FIG. 3G.

FIG. 3H.

FIG. 5
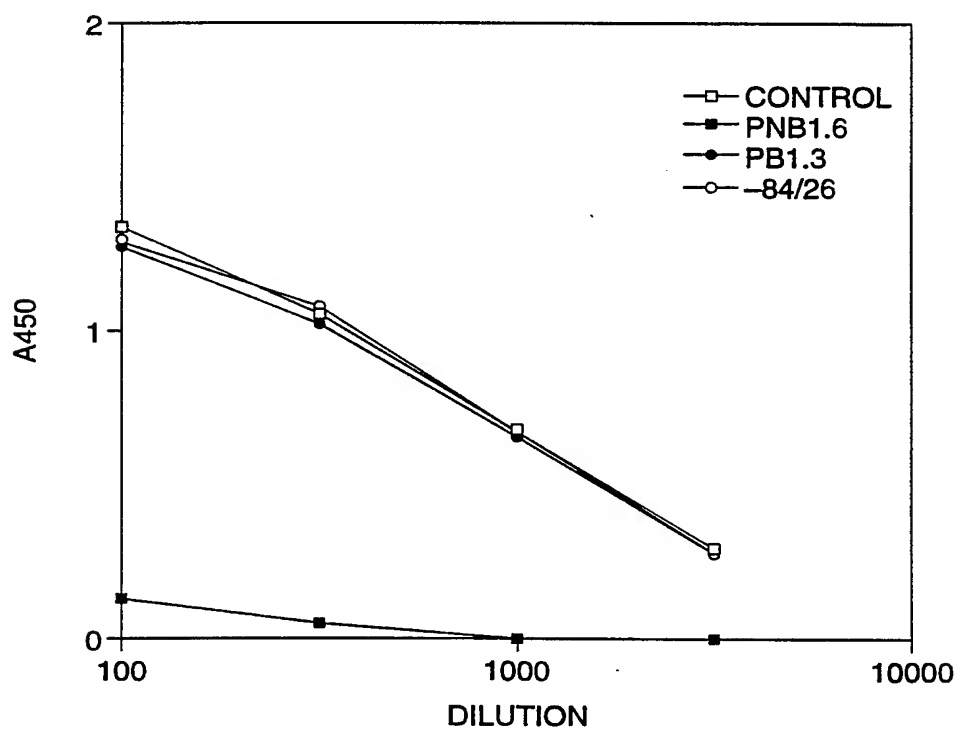
FIG. 6A
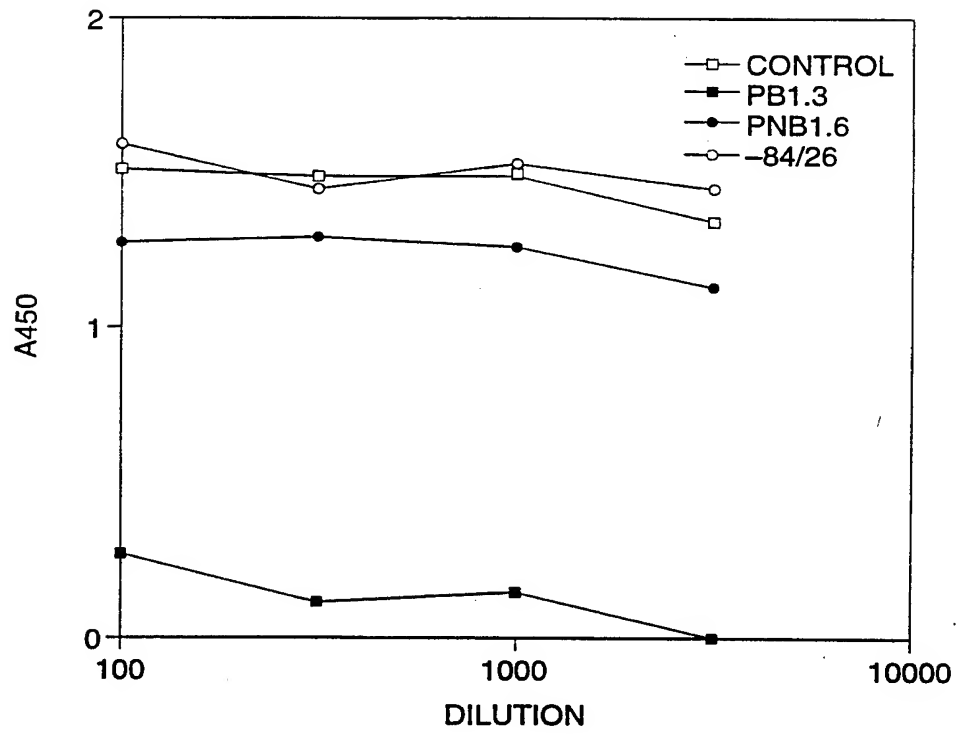
FIG. 6B

FIG. 6C

9/31
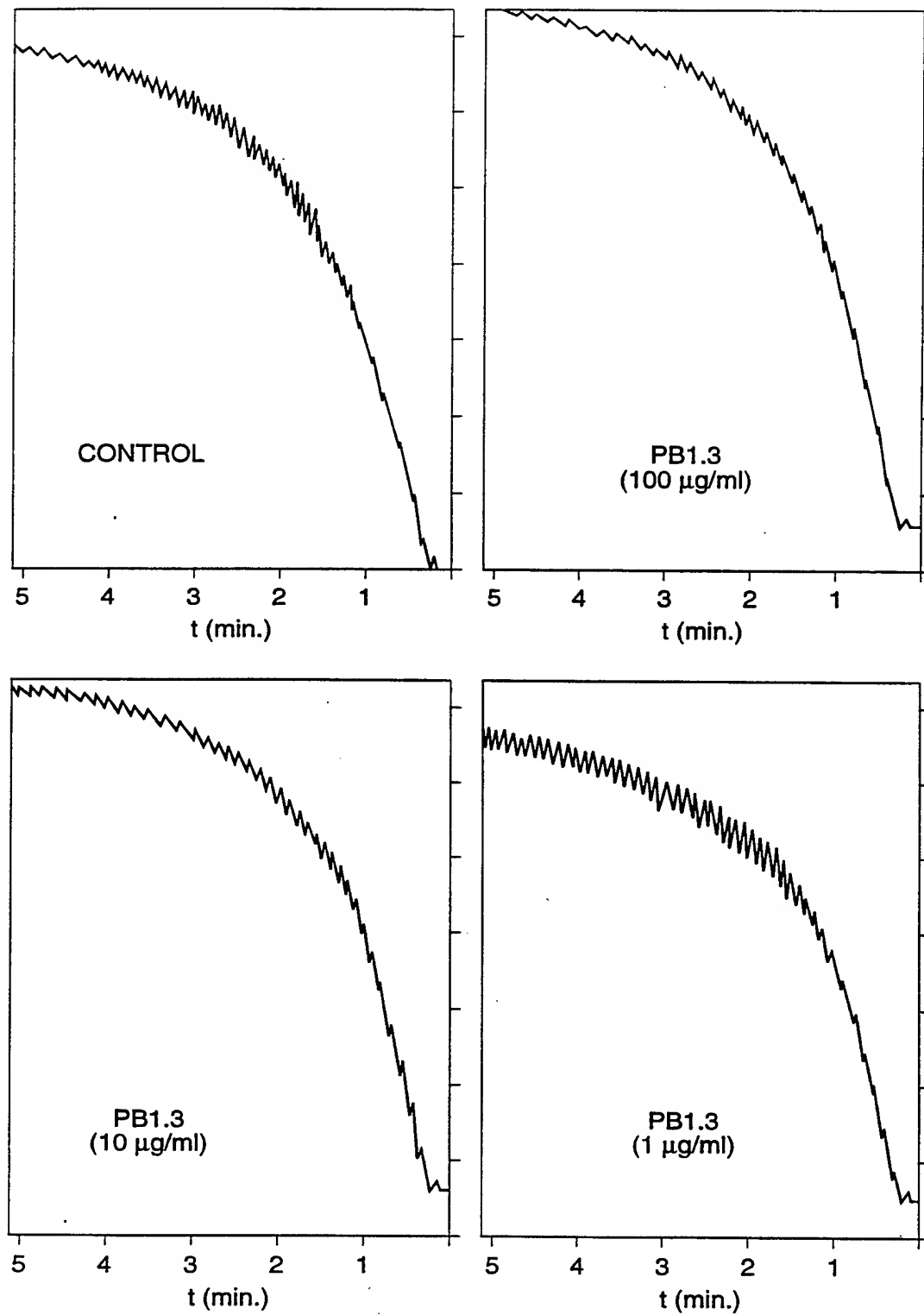
FIG. 7
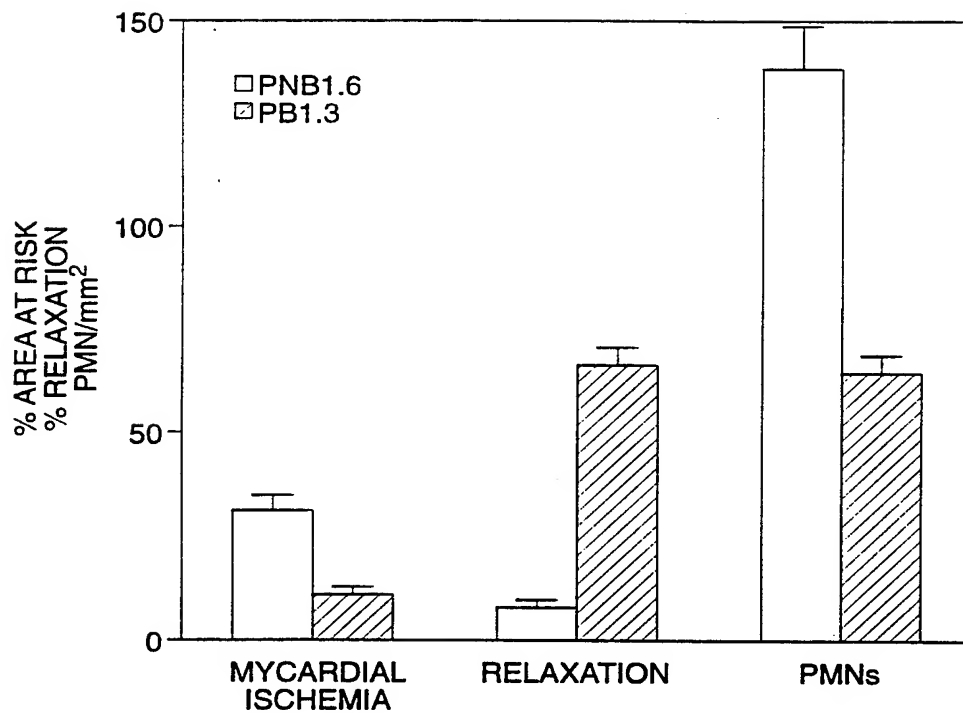
FIG. 8
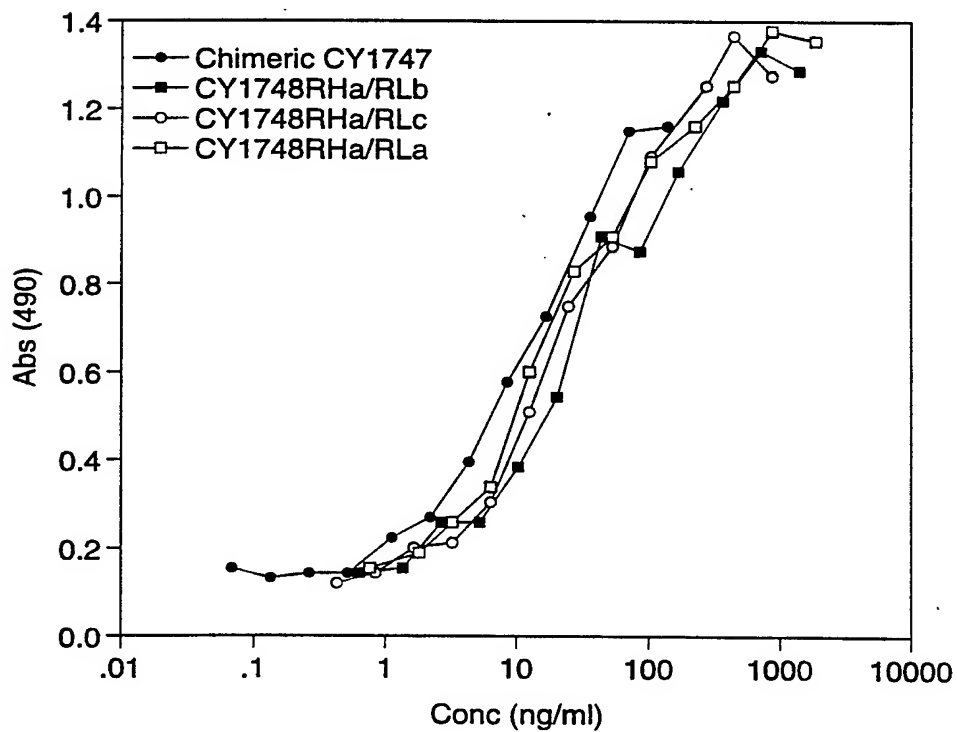
FIG. 20

```
                                                                48
                                                                 *
ATG GGA TGG AGC GGG GTC TTT CTC TTT CTC CTG TCA GGA ACT GCA GGT
Met Gly Trp Ser Gly Val Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly>


                                                                96
                                                                 *
SIGNAL SEQUENCE _ MATURE VARIABLE REGION SEQUENCE
GTC CAC TCT GAG GCC CAG CTG CAG CAG TCT GGA CCT GAG CTG GTA GAG
Val His Ser Glu Ala Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Glu>


                                                                144
                                                                 *
CCT GGG GCT TCA GTG AAG GTG TCC TGC AAG GCT TCT GGA TAC ACA TTC
Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe>


                                                                192
                                                                 *
ACC AAC TAT GTT ATG CAC TGG GTG AAG CAG AAG CCT GGG CAG GGC CTT
Thr Asn Tyr Val Met His Trp Val Lys Gln Lys Pro Gly Gln Gly Leu>


                                                                240
                                                                 *
GAG TGG ATT GGA TTT ATT AAC CCA TCC AAT GAT GGT CCT AAG TAC AAT
Glu Trp Ile Gly Phe Ile Asn Pro Ser Asn Asp Gly Pro Lys Tyr Asn>


                                                                288
                                                                 *
GAG AGG TTC AAA AAC AAG GCC ACA CTG ACT TCA GAC AAA TCC TCC AGC
Glu Arg Phe Lys Asn Lys Ala Thr Leu Thr Ser Asp Lys Ser Ser Ser>


                                                                336
                                                                 *
ACA GCC TAC ATG GAG CTC AGC AGC CTG ACC TCT GAG GTC TCT GCG GTC
Thr Ala Tyr Met Glu Leu Ser Ser Leu Thr Ser Glu Val Ser Ala Val>


                                                                384
                                                                 *
TAT TTC TGT GCA AGA GCC CGC CCG GGG TTC GAC TGG TAC TTC GAT GTC
Tyr Phe Cys Ala Arg Ala Arg Pro Gly Phe Asp Trp Tyr Phe Asp Val>


TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC TCA
Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ser
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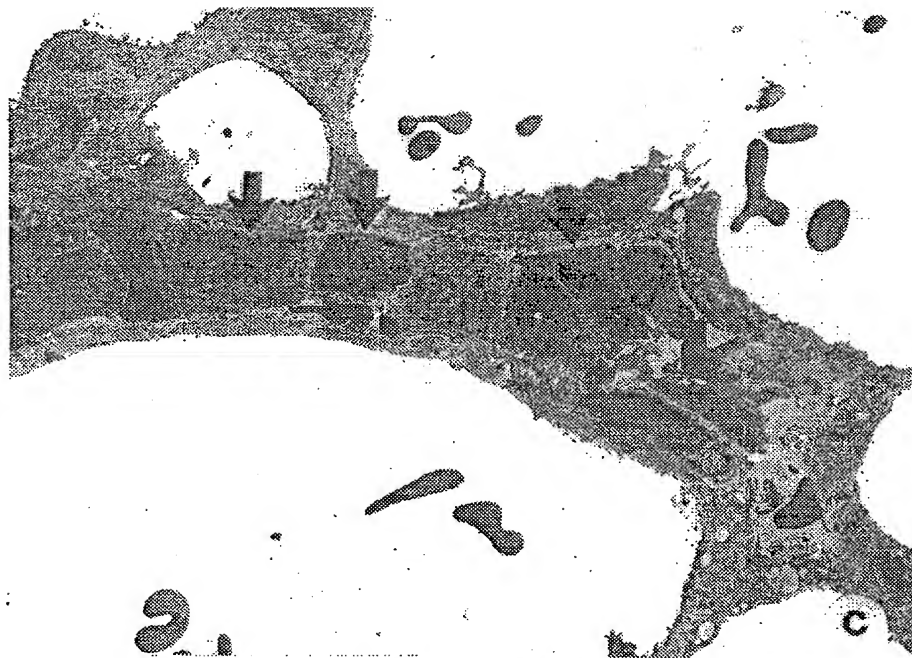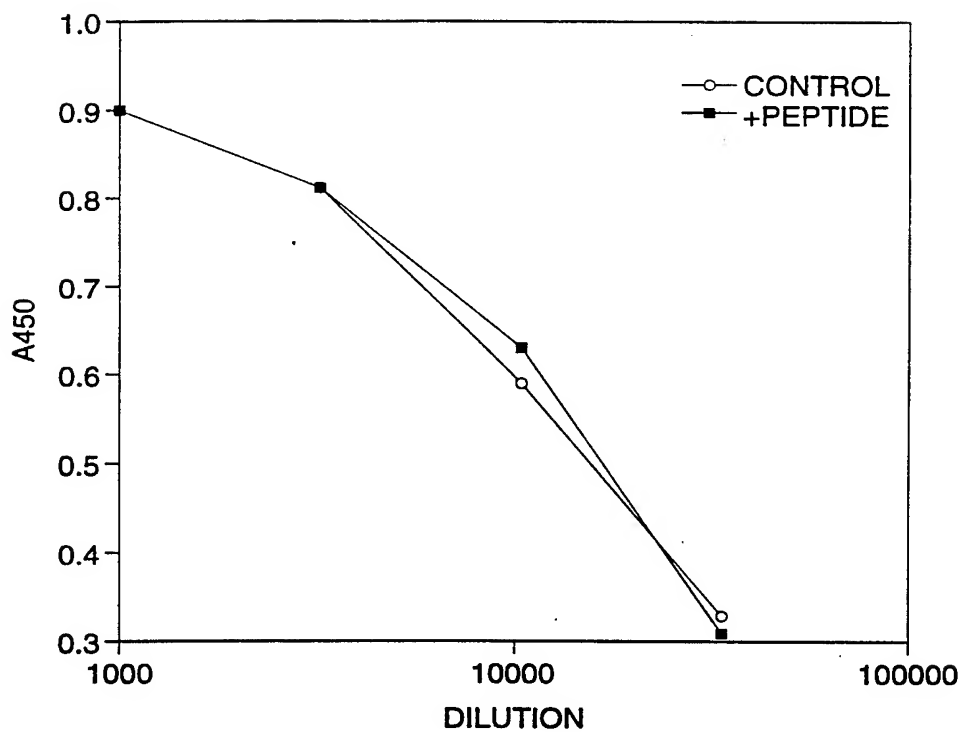
FIG. 9

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                                                                      48
                                                                       *
ATG GGC ATC AAG ATG GAG TCA CAG ATT CAG GTC TTT GTA TAC ATG TTG
Met Gly Ile Lys Met Glu Ser Gln Ile Gln Val Phe Val Tyr Met Leu>

                                                                      96
                 SIGNAL  SEQUENCE _ MATURE VARIABLE  REGION SEQ.      *
CTG TGG TTG TCT GGT GTT GAT GGA GAC ATT GTG ATG ACC CAG TCT CAA
Leu Trp Leu Ser Gly Val Asp Gly Asp Ile Val Met Thr Gln Ser Gln>

                                                                     144
                                                                       *
AAA TTC ATG TCC ACA TCA GTA GGA GAC AGG GTC AGC GTC ACC TGC AAG
Lys Phe Met Ser Thr Ser Val Gly Asp Arg Val Ser Val Thr Cys Lys>

                                                                     192
                                                                       *
GCC AGT CAG AAT GTG GCT ACT AAT GTA GTC TGG TAT CAA CAG AGA CCA
Ala Ser Gln Asn Val Ala Thr Asn Val Val Trp Tyr Gln Gln Arg Pro>

                                                                     240
                                                                       *
GGA CAA TCT CCT AAA GCG CTT ATT TAT ACG GCA TCC TAC CGG TTC AGT
Gly Gln Ser Pro Lys Ala Leu Ile Tyr Thr Ala Ser Tyr Arg Phe Ser>

                                                                     288
                                                                       *
GGA GTC CCT GAA CGC TTC TCA GGC AGT GGA TCT GGG ACA GAT TTC ACT
Gly Val Pro Glu Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr>

                                                                     336
                                                                       *
CTC ACC ATC ACC AAT GTG CAG TCT GAA GAC TTG GCA GAC TAT TTC TGT
Leu Thr Ile Thr Asn Val Gln Ser Glu Asp Leu Ala Asp Tyr Phe Cys>

                                                                     384
                                                                       *
CAA CAA TAT AAC AAC TAT CCC TAC ACG TTC GGA GGG GGG ACC AAG GTG
Gln Gln Tyr Asn Asn Tyr Pro Tyr Thr Phe Gly Gly Gly Thr Lys Val>

GAA ATT CAA
Glu Ile Gln
```
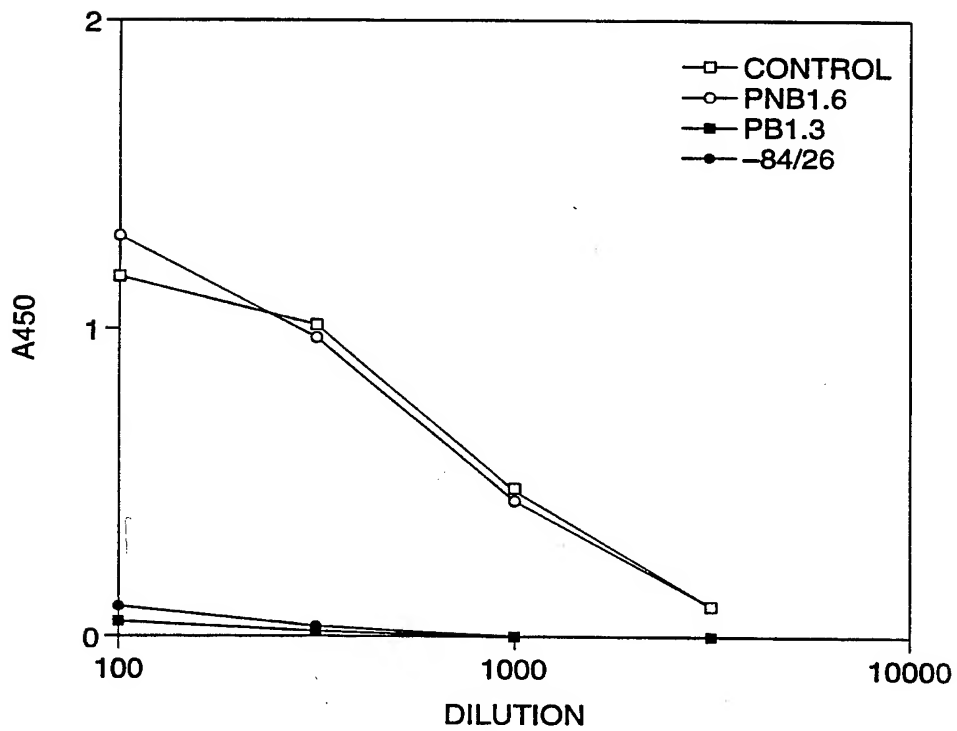
FIG. 10

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                                                                    48
                                                                    *
        ATG GAG TTT GGG CTG AGC TGG CTT TTT CTT GTG GCT ATT TTA AAA GGT
        Met Glu Phe Gly Leu Ser Trp Leu Phe Leu Val Ala Ile Leu Lys Gly>


                                                                    96
SIGNAL SEQUENCE _ MATURE  VARIABLE REGION  SEQUENCE                  *
        GTC CAG TGT GAG GCC CAG CTG GTG CAG TCT GGA GCT GAG GTG AAA AAG
        Val Gln Cys Glu Ala Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys>


                                                                    144
                                                                    *
        CCT GGG GCT TCA GTG AAG GTG TCC TGC AAG GCT TCT GGA TAC ACA TTC
        Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe>


                                                                    192
                                                                    *
        ACC AAC TAT GTT ATG CAC TGG GTG CGC CAA GCT CCC GGG CAG AGG CTT
        Thr Asn Tyr Val Met His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu>


                                                                    240
                                                                    *
        GAG TGG ATG GGA TTT ATT AAC CCA TCC AAT GAT GGT CCT AAG TAC AAT
        Glu Trp Met Gly Phe Ile Asn Pro Ser Asn Asp Gly Pro Lys Tyr Asn>
                                                                    288
                                                                    *
        GAG AGG TTC AAA AAC AGG GTC ACA ATC ACT TCA GAC ACA TCC GCC AGC
        Glu Arg Phe Lys Asn Arg Val Thr Ile Thr Ser Asp Thr Ser Ala Ser>


                                                                    336
                                                                    *
        ACC GCC TAC ATG GAA CTG TCC AGC CTG CGC TCC GAG GAC ACT GCA GTC
        Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val>


                                                                    384
                                                                    *
        TAT TAC TGT GCC AGA GCA CGC CCG GGG TTC GAC TGG TAC TTC GAT GTC
        Tyr Tyr Cys Ala Arg Ala Arg Pro Gly Phe Asp Trp Tyr Phe Asp Val>


        TGG GGA CAG GGT ACC CTT GTC ACC GTC AGT TCA
        Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
```

FIG. 11

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                                                                        48
                                                                        *
        ATG GAG TTT GGG CTG AGC TGG CTT TTT CTT GTG GCT ATT TTA AAA GGT
        Met Glu Phe Gly Leu Ser Trp Leu Phe Leu Val Ala Ile Leu Lys Gly>


                                                                        96
                                                                        *
SIGNAL SEQUENCE _ MATURE VARIABLE REGION SEQUENCE
        GTC CAG TGT CAG GTC CAG CTG GTG CAG TCT GGA GCT GAG GTG AAA AAG
        Val Gln Cys Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys>


                                                                       144
                                                                        *
        CCT GGG GCT TCA GTG AAG GTG TCC TGC AAG GCT TCT GGA TAC ACA TTC
        Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe>


                                                                      ˙192
                                                                        *
        ACC AAC TAT GTT ATG CAC TGG GTG CGC CAA GCT CCC GGG CAG AGG CTT
        Thr Asn Tyr Val Met His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu>


                                                                       240
                                                                        *
        GAG TGG ATG GGA TTT ATT AAC CCA TCC AAT GAT GGT CCT AAG TAC AAT
        Glu Trp Met Gly Phe Ile Asn Pro Ser Asn Asp Gly Pro Lys Tyr Asn>
                                                                       288
                                                                        *
        GAG AGG TTC AAA AAC AGG GTC ACA ATC ACT TCA GAC ACA TCC GCC AGC
        Glu Arg Phe Lys Asn Arg Val Thr Ile Thr Ser Asp Thr Ser Ala Ser>


                                                                       336
                                                                        *
        ACC GCC TAC ATG GAA CTG TCC AGC CTG CGC TCC GAG GAC ACT GCA GTC
        Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val>


                                                                       384
                                                                        *
        TAT TAC TGT GCC AGA GCA CGC CCG GGG TTC GAC TGG TAC TTC GAT GTC
        Tyr Tyr Cys Ala Arg Ala Arg Pro Gly Phe Asp Trp Tyr Phe Asp Val>

        TGG GGA CAG GGT ACC CTT GTC ACC GTC AGT TCA
        Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
```

FIG. 12

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                                                                    48
                                                                     *
    ATG GAG TTT GGG CTG AGC TGG CTT TTT CTT GTG GCT ATT TTA AAA GGT
    Met Glu Phe Gly Leu Ser Trp Leu Phe Leu Val Ala Ile Leu Lys Gly>

                                                                    96
SIGNAL SEQUENCE _ MATURE VARIABLE REGION SEQUENCE                    *
    GTC CAG TGT GAG GCC CAG CTG GTG CAG TCT GGA GCT GAG GTG AAA AAG
    Val Gln Cys Glu Ala Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys>

                                                                   144
                                                                     *
    CCT GGG GCT TCA GTG AAG GTG TCC TGC AAG GCT TCT GGA TAC ACA TTC
    Pro Gly Ala Ser Val Lys Val Ser Cys Lys Ala Ser Gly Tyr Thr Phe>

                                                                   192
                                                                     *
    ACC AAC TAT GTT ATG CAC TGG GTG CGC CAA GCT CCC GGG CAG AGG CTT
    Thr Asn Tyr Val Met His Trp Val Arg Gln Ala Pro Gly Gln Arg Leu>

                                                                   240
                                                                     *
    GAG TGG ATG GGA TTT ATT AAC CCA TCC AAT GAT GGT CCT AAG TAC AAT
    Glu Trp Met Gly Phe Ile Asn Pro Ser Asn Asp Gly Pro Lys Tyr Asn>

                                                                   288
                                                                     *
    GAG AGG TTC AAA AAC AGG GTC ACA ATC ACT TCA GAC AAA TCC GCC AGC
    Glu Arg Phe Lys Asn Arg Val Thr Ile Thr Ser Asp Lys Ser Ala Ser>

                                                                   336
                                                                     *
    ACC GCC TAC ATG GAA CTG TCC AGC CTG CGC TCC GAG GAC ACT GCA GTC
    Thr Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val>

                                                                   384
                                                                     *
    TAT TAC TGT GCC AGA GCA CGC CCG GGG TTC GAC TGG TAC TTC GAT GTC
    Tyr Tyr Cys Ala Arg Ala Arg Pro Gly Phe Asp Trp Tyr Phe Asp Val>

    TGG GGA CAG GGT ACC CTT GTC ACC GTC AGT TCA
    Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
```

FIG. 13

```
                                                                          48
                                                                           *
        ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT
        Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly>


                                                                          96
SIGNAL SEQUENCE _ MATURE VARIABLE REGION SEQUENCE                          *
        GTC CAC TCC GAC ATC CAG ATG ACC CAG AGC CCA TCC ACC CTG TCT GCA
        Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser Ala>


                                                                         144
                                                                           *
        AGC GTA GGA GAC AGA GTC ACC GTC ACC TGC AAG GCC AGT CAG AAT GTG
        Ser Val Gly Asp Arg Val Thr Val Thr Cys Lys Ala Ser Gln Asn Val>


                                                                         192
                                                                           *
        GCT ACT AAT GTA GTC TGG TAT CAA CAG AAA CCA GGA GAG GCT CCT AAA
        Ala Thr Asn Val Val Trp Tyr Gln Gln Lys Pro Gly Glu Ala Pro Lys>


                                                                         240
                                                                           *
        GCG CTT ATT TAT ACG GCA TCC TAC CGG TTC AGT GGA GTC CCT GAA CGC
        Ala Leu Ile Tyr Thr Ala Ser Tyr Arg Phe Ser Gly Val Pro Glu Arg>


                                                                         288
                                                                           *
        TTC TCA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGT
        Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser>


                                                                         336
                                                                           *
        CTG CAG TCT GAT GAC TTT GCA ACT TAT TAC TGT CAA CAG TAT AAC AAT
        Leu Gln Ser Asp Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Asn>


        TAC CCA TAC ACG TTC GGC CAA GGG ACC AAG CTC GAA ATC AAA
        Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
```

FIG. 14

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```
                                                                    48
                                                                     *
        ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT
        Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly>


                                                                    96
SIGNAL SEQUENCE _ MATURE VARIABLE REGION  SEQUENCE                    *
        GTC CAC TCC GAC ATC CAG ATG ACC CAG AGC CCA TCC ACC CTG TCT GCA
        Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser Ala>


                                                                    144
                                                                     *
        AGC GTA GGA GAC AGA GTC ACC GTC ACC TGC AAG GCC AGT CAG AAT GTG
        Ser Val Gly Asp Arg Val Thr Val Thr Cys Lys Ala Ser Gln Asn Val>


                                                                    192
                                                                     *
        GCT ACT AAT GTA GTC TGG TAT CAA CAG AAA CCA GGA GAG GCT CCT AAA
        Ala Thr Asn Val Val Trp Tyr Gln Gln Lys Pro Gly Glu Ala Pro Lys>


                                                                    240
                                                                     *
        GCG CTT ATT TAT ACG GCA TCC TAC CGG TTC AGT GGA GTC CCT TCA CGC
        Ala Leu Ile Tyr Thr Ala Ser Tyr Arg Phe Ser Gly Val Pro Ser Arg>


                                                                    288
                                                                     *
        TTC TCA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGT
        Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser>


                                                                    336
                                                                     *
        CTG CAG TCT GAT GAC TTT GCA ACT TAT TAC TGT CAA CAG TAT AAC AAT
        Leu Gln Ser Asp Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Asn>


        TAC CCA TAC ACG TTC GGC CAA GGG ACC AAG CTC GAA ATC AAA
        Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
```

FIG. 15

```
                                                                        48
                                                                         *
    .ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT
     Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly>


                                                                        96
SIGNAL SEQUENCE _ MATURE  VARIABLE REGION  SEQUENCE                       *
     GTC CAC TCC GAC ATC CAG ATG ACC CAG AGC CCA TCC ACC CTG TCT GCA
     Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser Ala>


                                                                       144
                                                                         *
     AGC GTA GGA GAC AGA GTC ACC GTC ACC TGC AAG GCC AGT CAG AAT GTG
     Ser Val Gly Asp Arg Val Thr Val Thr Cys Lys Ala Ser Gln Asn Val>


                                                                       192
                                                                         *
     GCT ACT AAT GTA GTC TGG TAT CAA CAG AAA CCA GGA GAG GCT CCT AAA
     Ala Thr Asn Val Val Trp Tyr Gln Gln Lys Pro Gly Glu Ala Pro Lys>


                                                                       240
                                                                         *
     GCG CTT ATT TAT ACG GCA TCC TAC CGG TTC AGT GGA GTC CCT GAA CGC
     Ala Leu Ile Tyr Thr Ala Ser Tyr Arg Phe Ser Gly Val Pro Glu Arg>


                                                                       288
                                                                         *
     TTC TCA GGC AGT GGA TCT GGG ACA GAA TTC ACT CTC ACC ATC AGC AGT
     Phe Ser Gly Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser>


                                                                       336
                                                                         *
     CTG CAG TCT GAT GAC TTT GCA ACT TAT TAC TGT CAA CAG TAT AAC AAT
     Leu Gln Ser Asp Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Asn>


     TAC CCA TAC ACG TTC GGC CAA GGG ACC AAG CTC GAA ATC AAA
     Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
```
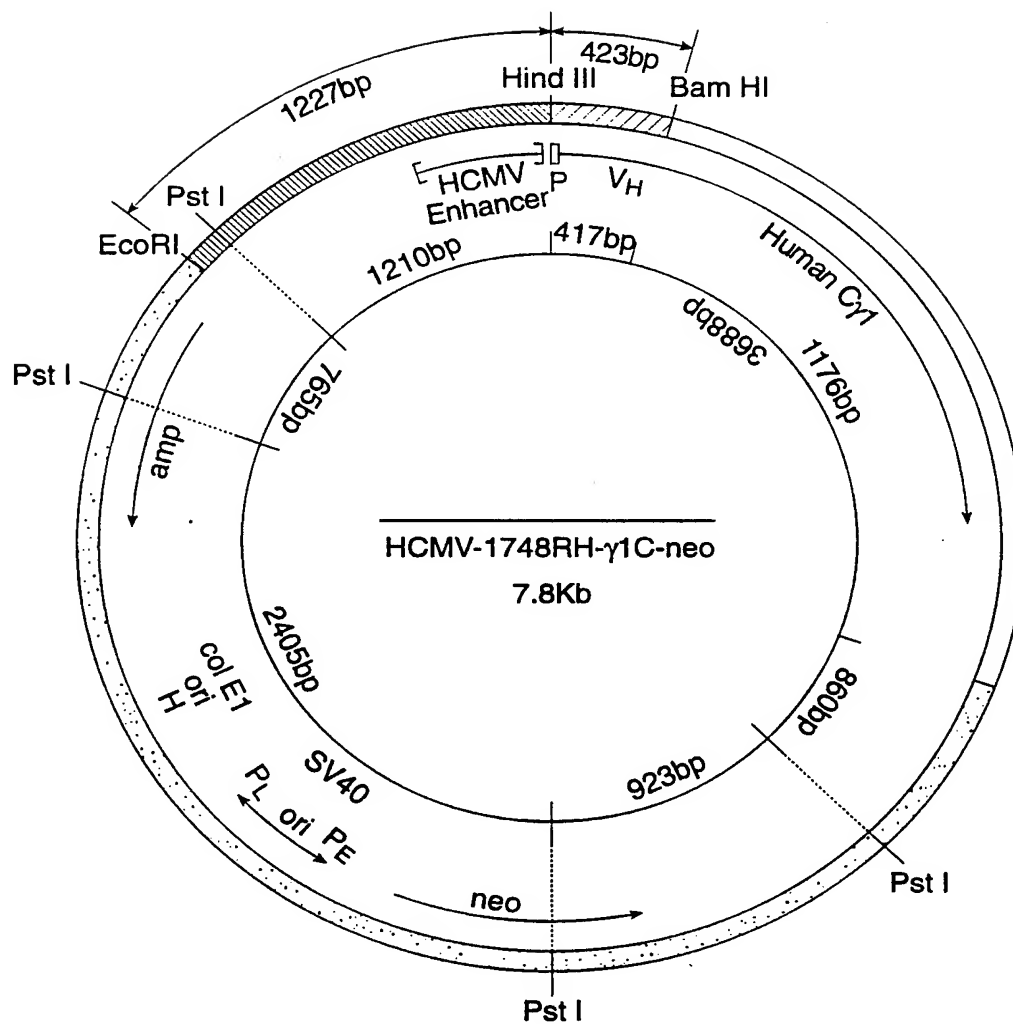
FIG. 16

```
                                                                    48
                                                                     *
   ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG GTA GCA ACA GCT ACA GGT
   Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly>

                                                                    96
SIGNAL SEQUENCE _ MATURE VARIABLE REGION SEQUENCE                    *
   GTC CAC TCC GAC ATC CAG ATG ACC CAG AGC CCA TCC ACC CTG TCT GCA
   Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Thr Leu Ser Ala>

                                                                   144
                                                                     *
   AGC GTA GGA GAC AGA GTC ACC GTC ACC TGC AAG GCC AGT CAG AAT GTG
   Ser Val Gly Asp Arg Val Thr Val Thr Cys Lys Ala Ser Gln Asn Val>

                                                                   192
                                                                     *
   GCT ACT AAT GTA GTC TGG TAT CAA CAG AAA CCA GGA GAG GCT CCT AAA
   Ala Thr Asn Val Val Trp Tyr Gln Gln Lys Pro Gly Glu Ala Pro Lys>

                                                                   240
                                                                     *
   GCG CTT ATT TAT ACG GCA TCC TAC CGG TTC AGT GGA GTC CCT TCA CGC
   Ala Leu Ile Tyr Thr Ala Ser Tyr Arg Phe Ser Gly Val Pro Ser Arg>

                                                                   288
                                                                     *
   TTC TCA GGC AGT GGA TCT GGG ACA GAA TTC ACT CTC ACC ATC AGC AGT
   Phe Ser Gly Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Ile Ser Ser>

                                                                   336
                                                                     *
   CTG CAG TCT GAT GAC TTT GCA ACT TAT TAC TGT CAA CAG TAT AAC AAT
   Leu Gln Ser Asp Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Asn>

   TAC CCA TAC ACG TTC GGC CAA GGG ACC AAG CTC GAA ATC AAA
   Tyr Pro Tyr Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
```

FIG. 17

☒ RESHAPED 1748RH VARIABLE REGION
☐ HUMAN γ-1 CONSTANT REGION (cDNA FRAGMENT)
▨ HCMV DNA FRAGMENT
⊡ pSV2neo VECTOR FRAGMENT

FIG. 18

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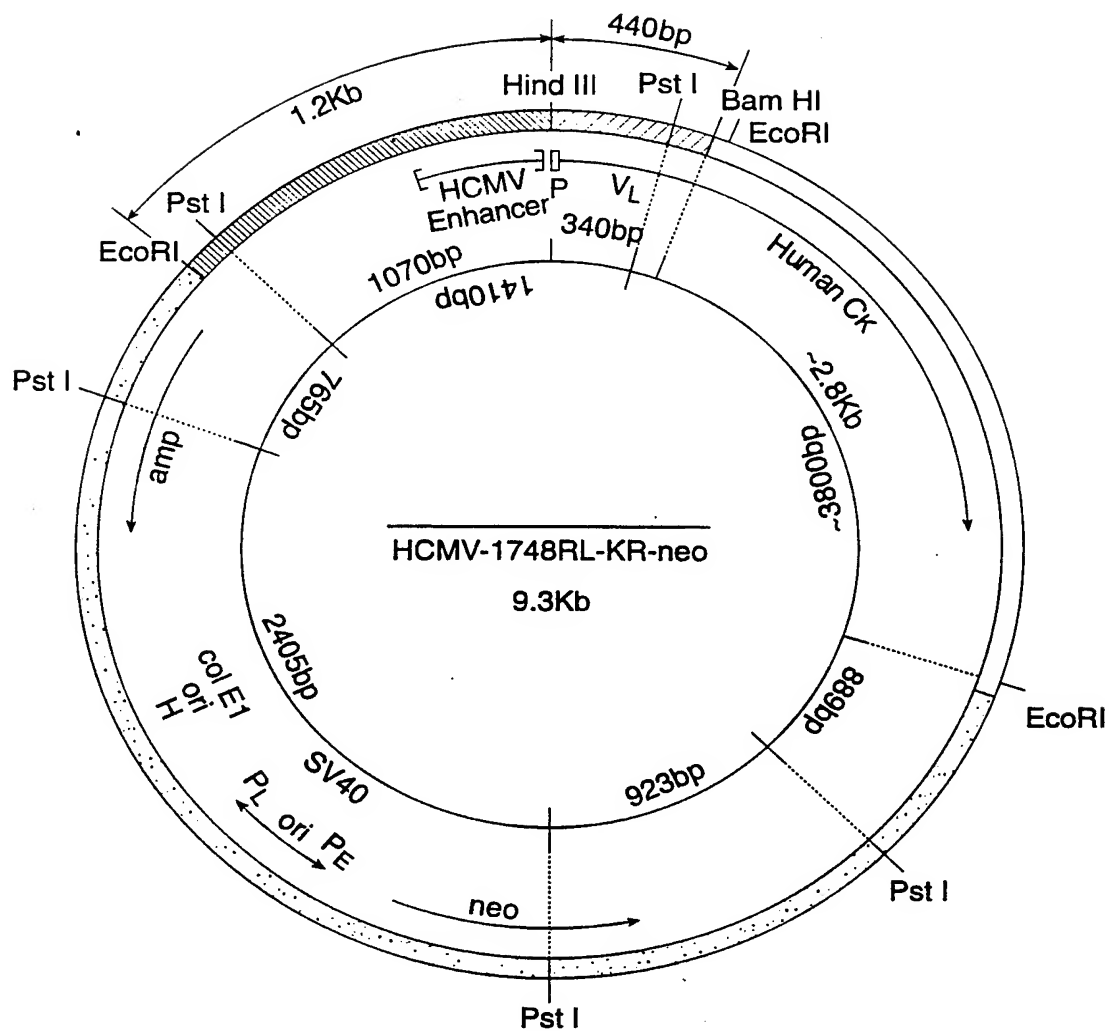
☑ RESHAPED 1748RL VARIABLE REGION
☐ HUMAN KAPPA CONSTANT REGION (GENOMIC DNA FRAGMENT)
▨ HCMV DNA FRAGMENT
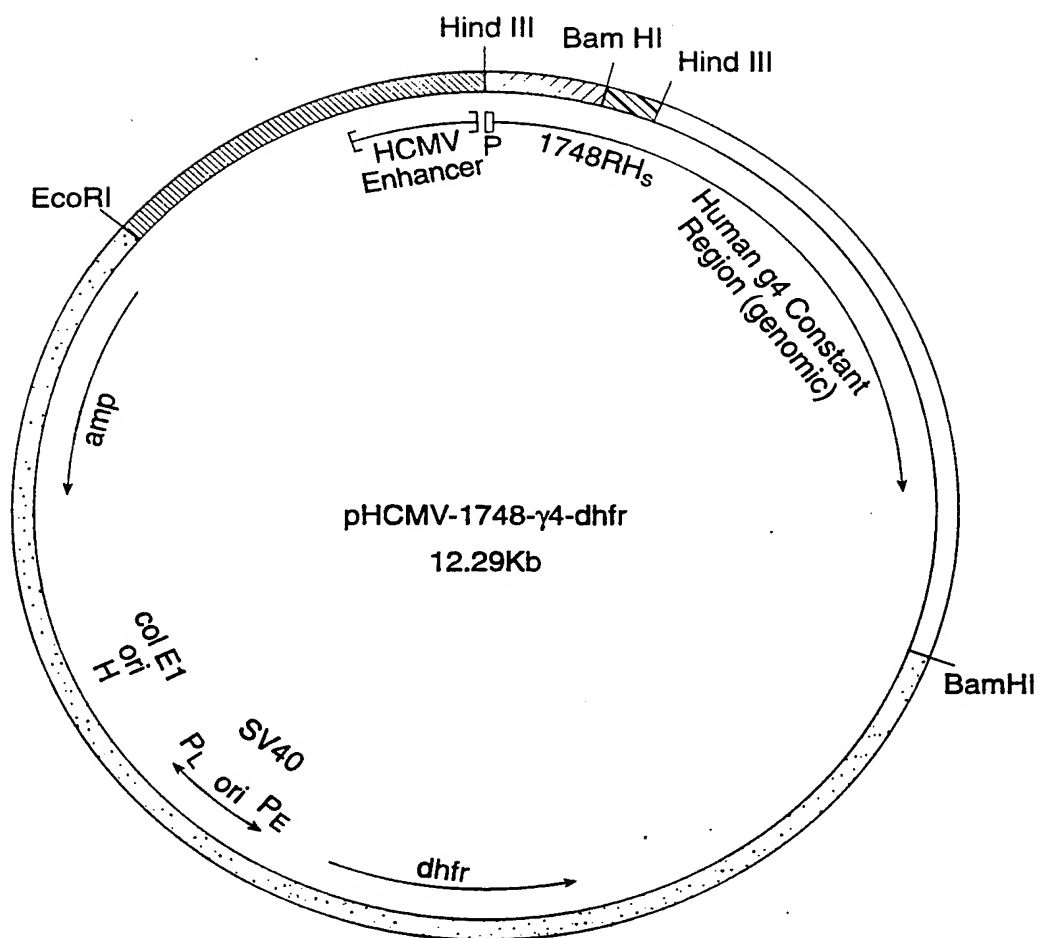☐ pSV2neo VECTOR FRAGMENT

FIG. 19
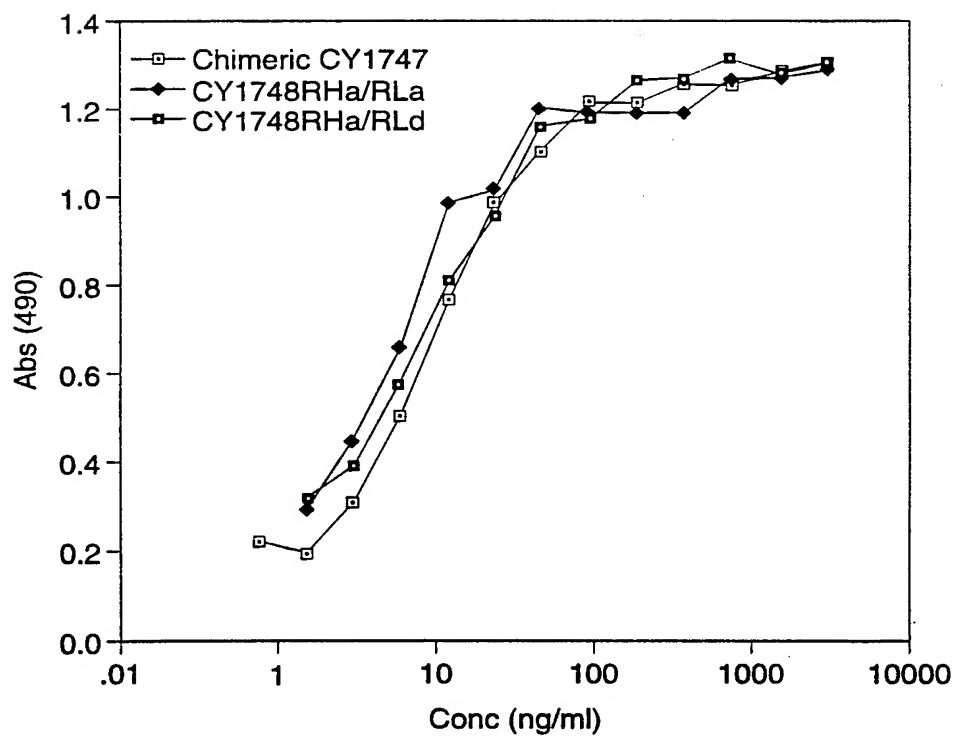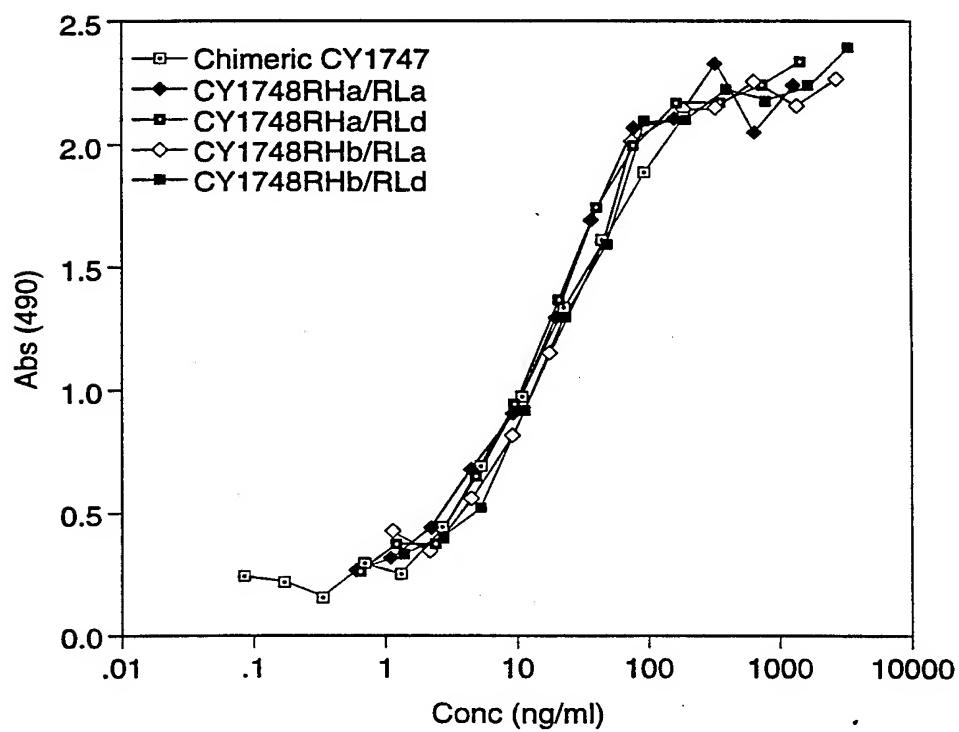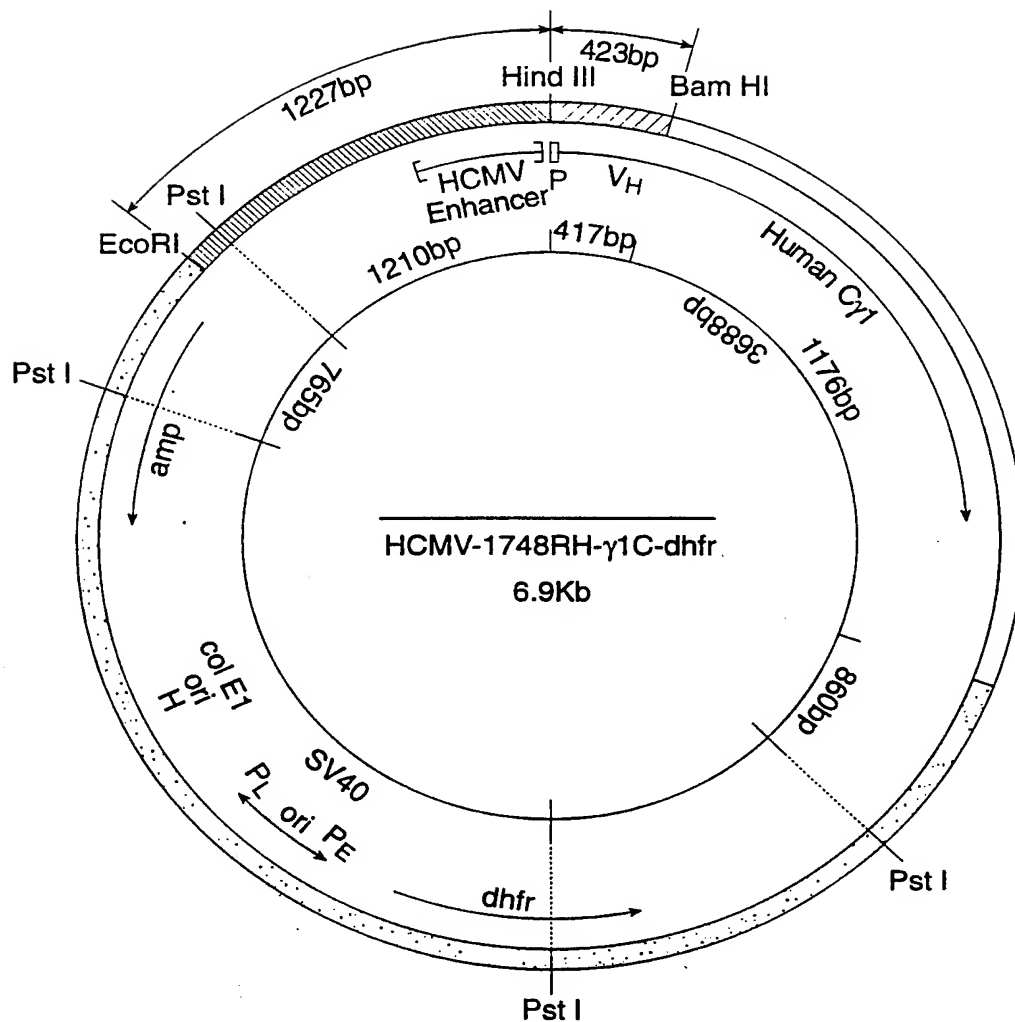
FIG. 21

FIG. 22

RESHAPED 1748RH VARIABLE REGION
HUMAN γ-1 CONSTANT REGION (cDNA FRAGMENT)
HCMV DNA FRAGMENT
pSV2neo VECTOR FRAGMENT

FIG. 23

RESHAPED 1748RH VARIABLE REGION
HUMAN γ-4 CONSTANT REGION (GENOMIC)
HCMV PROMOTER/ENHANCER
pSV2dhfr
MCS (BamHI-XbaI-SalI-PstI=HindIII)

FIG. 24
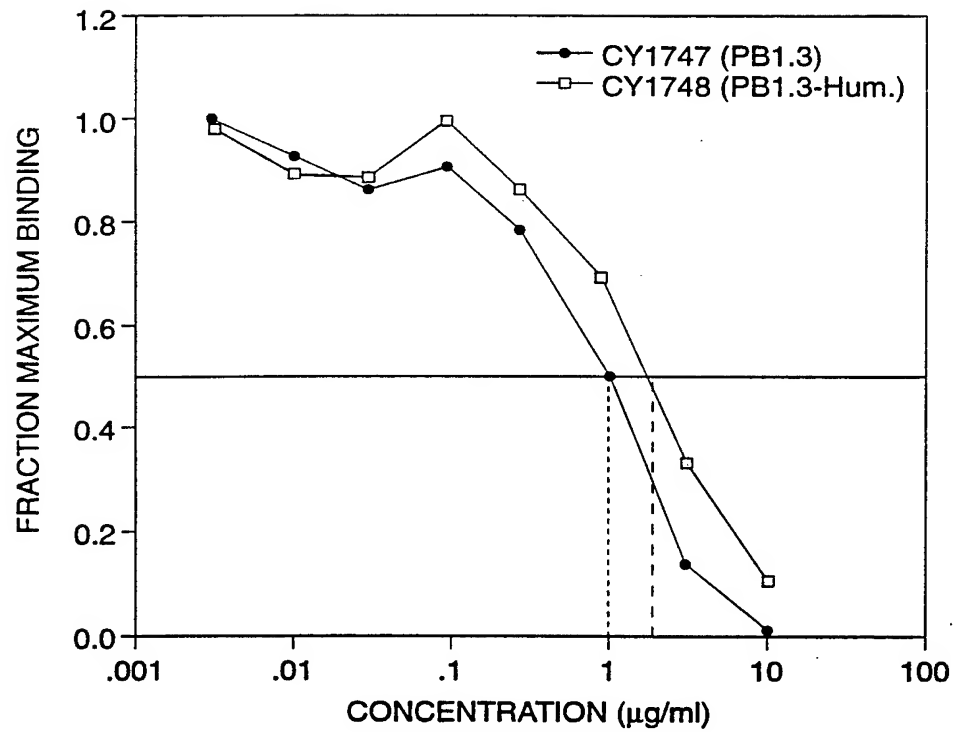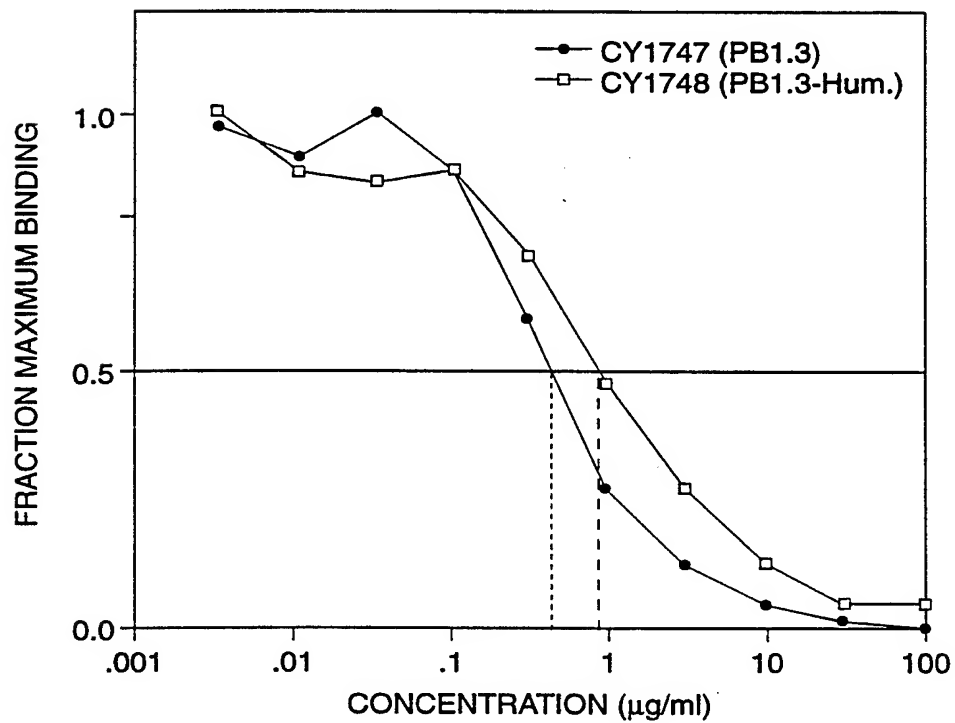
FIG. 25

FIG. 26
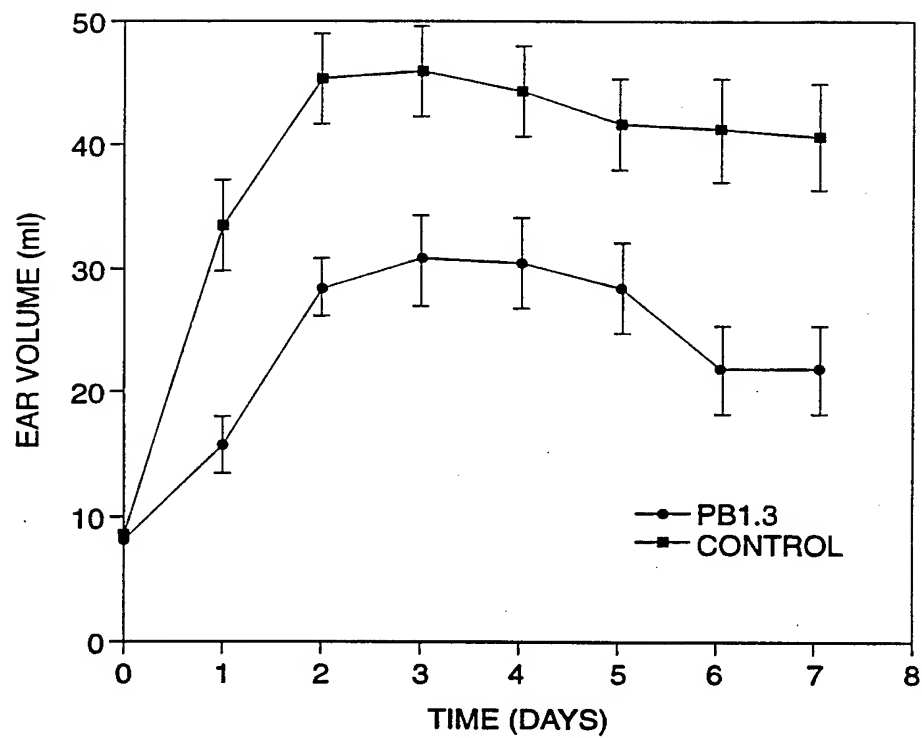
FIG. 27
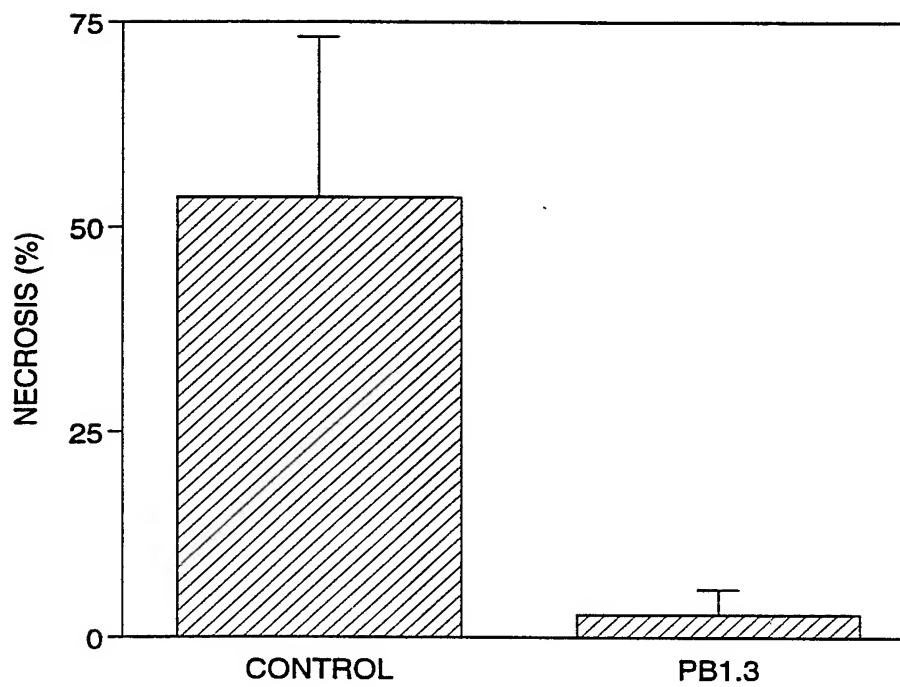
FIG. 28
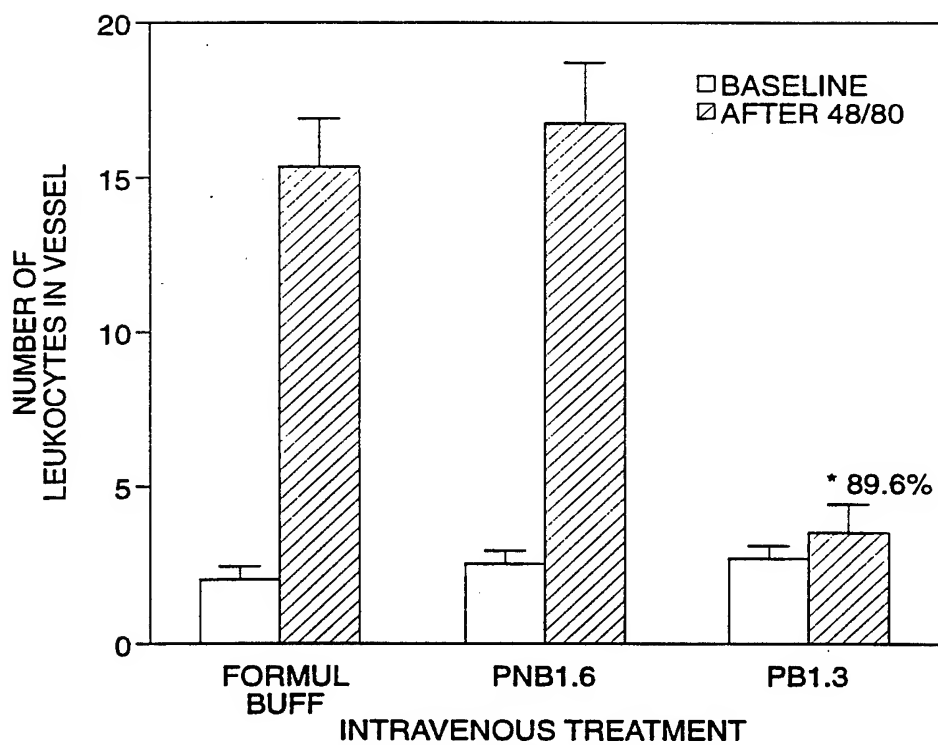
FIG. 29
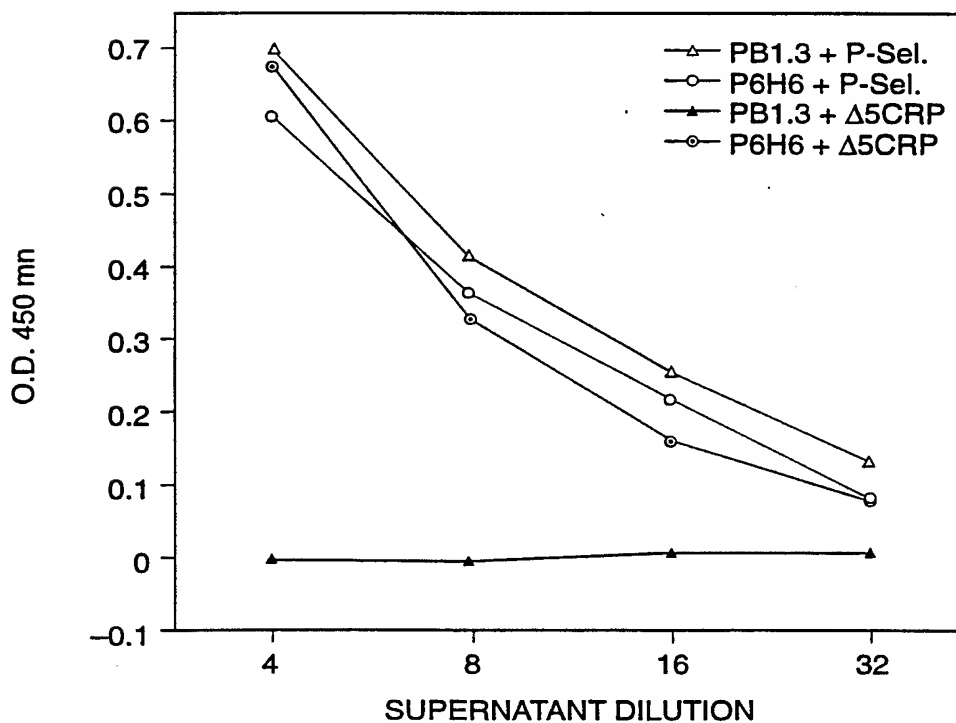
FIG. 33
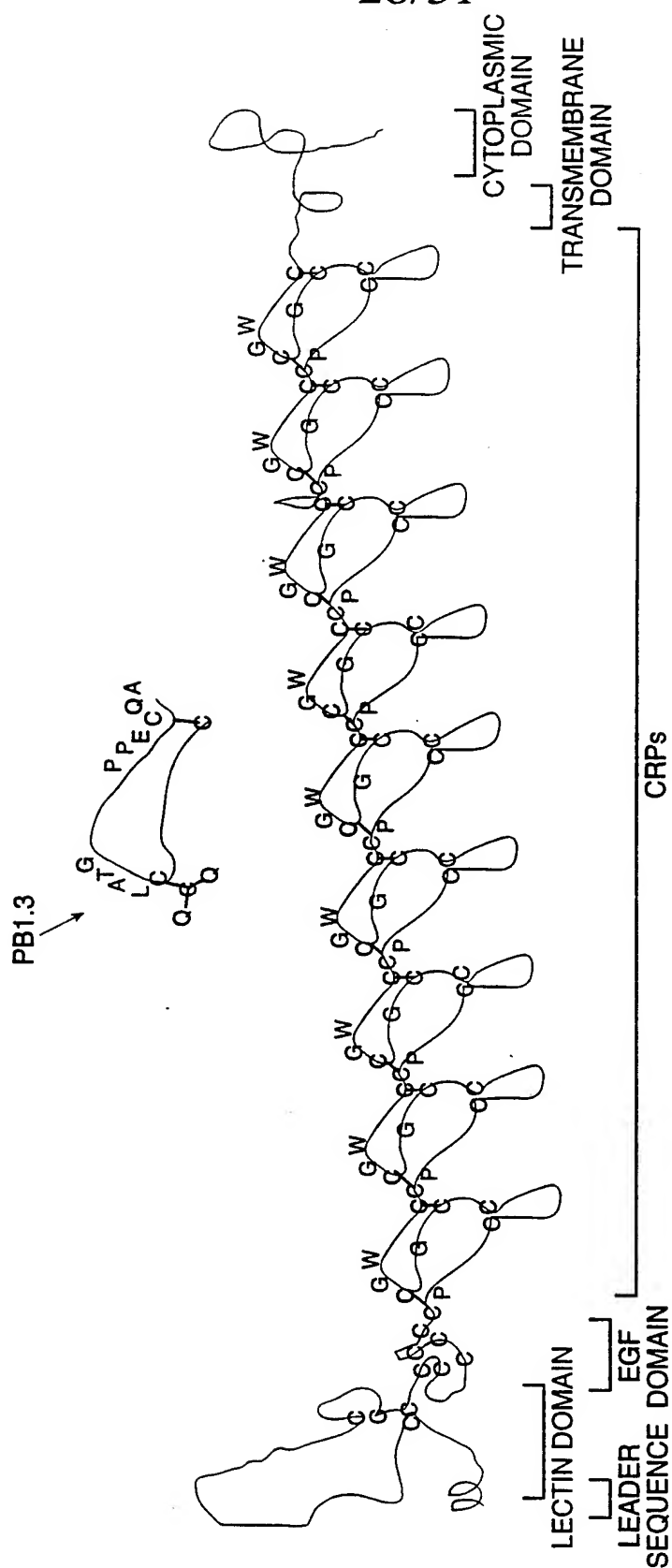
FIG. 30
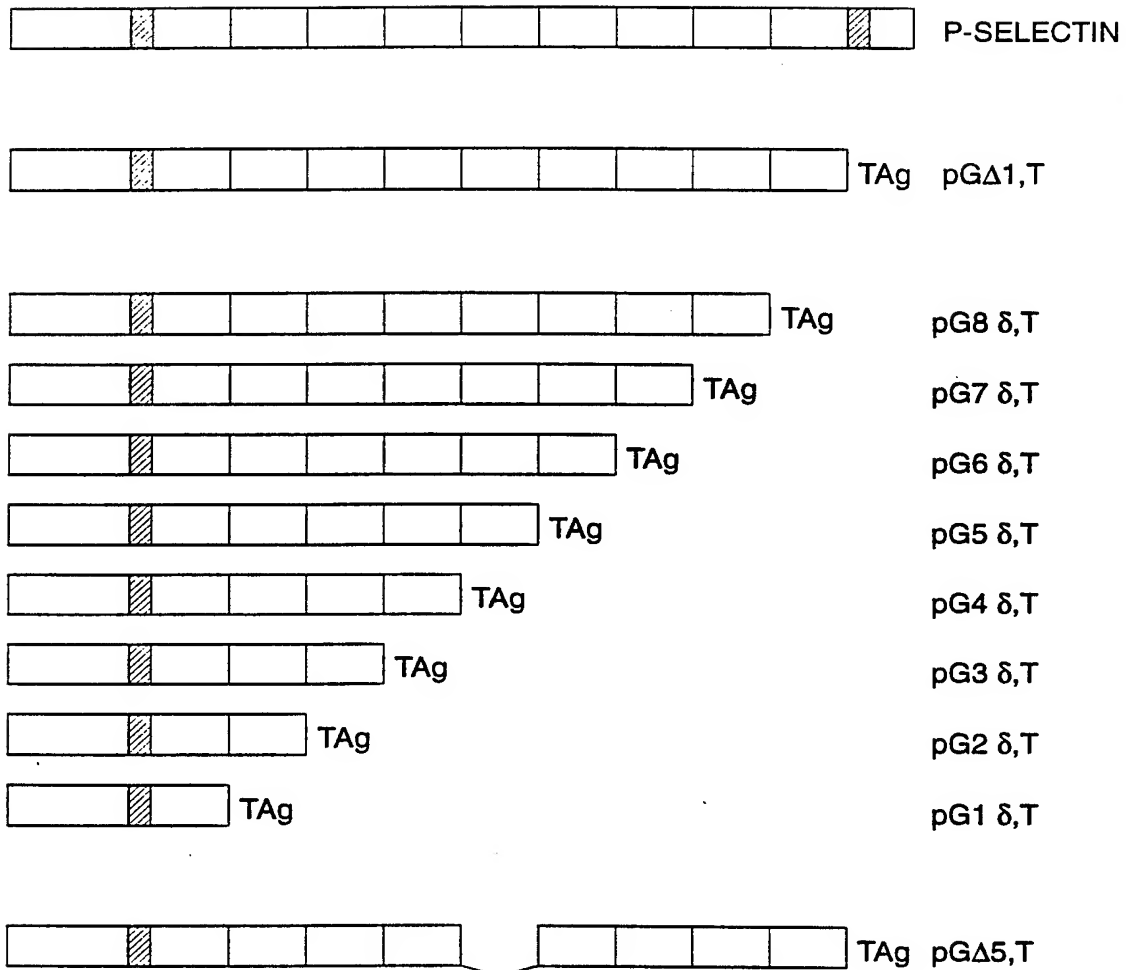
FIG. 31
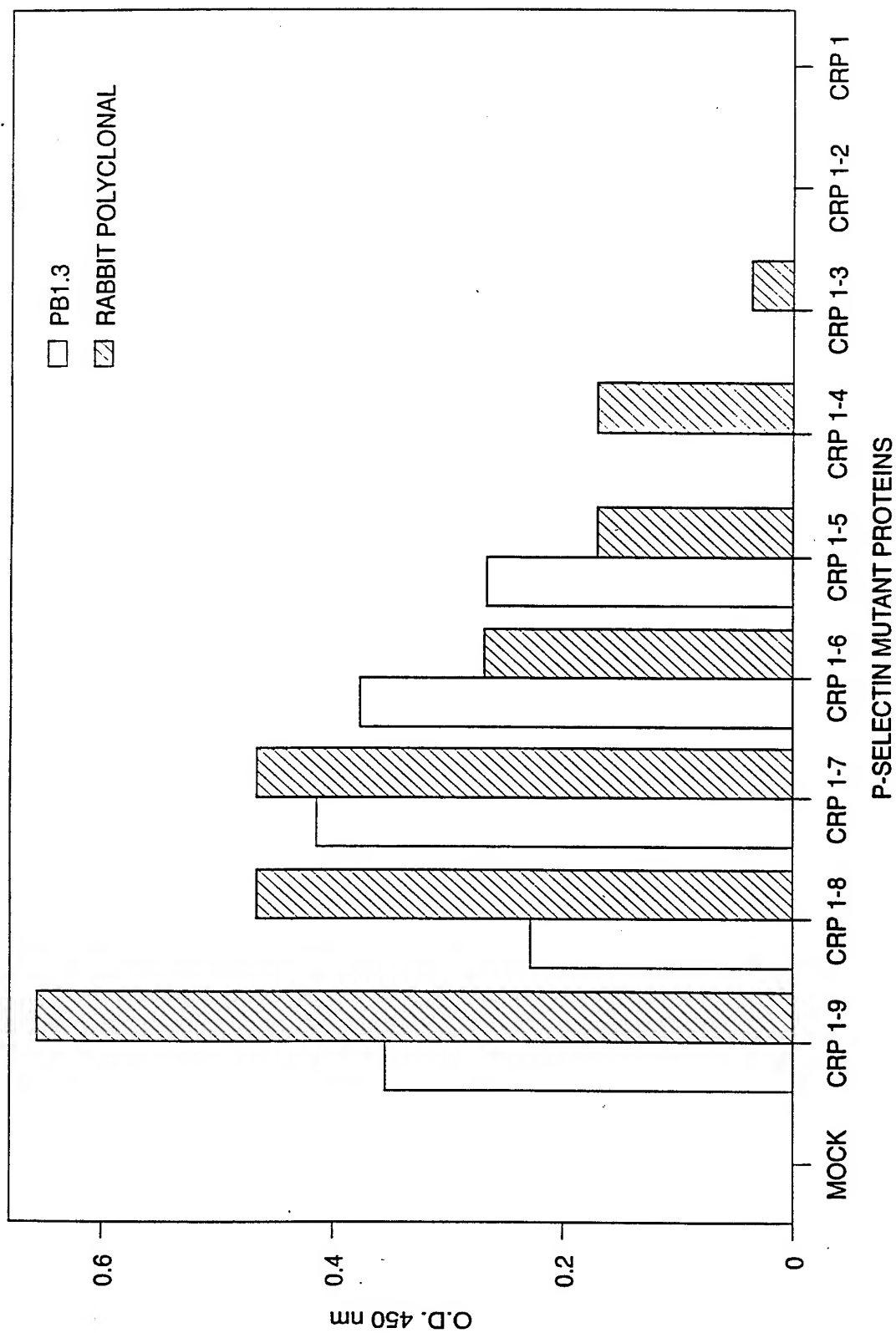
FIG. 32
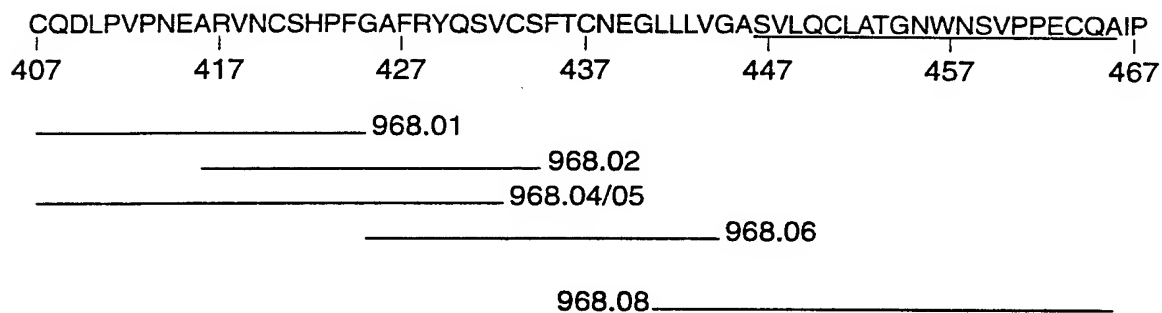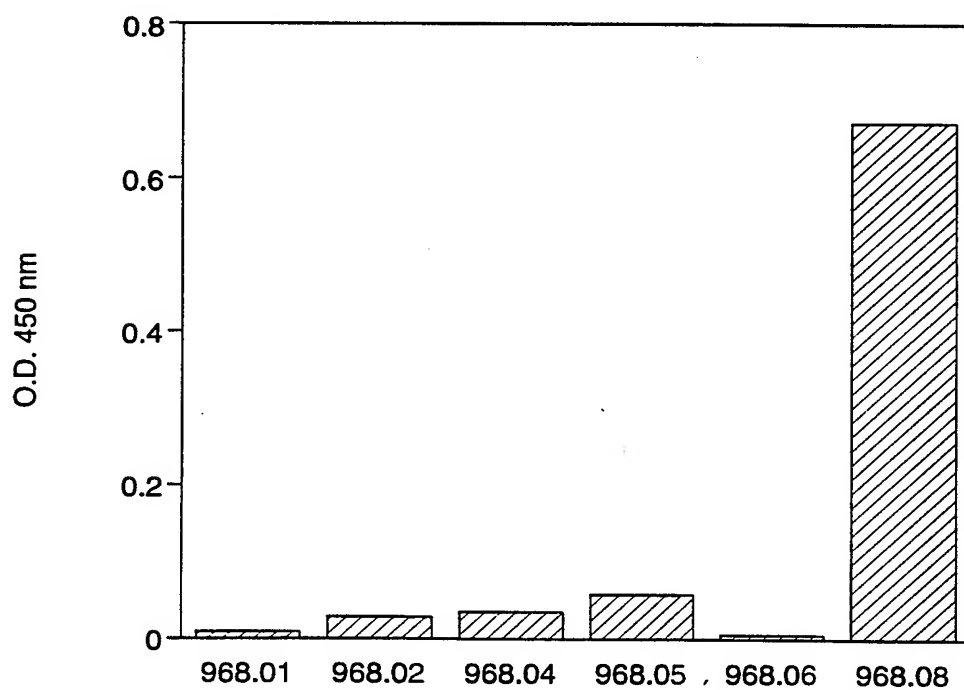
FIG. 34A

FIG. 34B

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/395; C12P 21/00; C12N 5/10, 7/01, 15/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8; 435/69.6, 172.3, 252.3, 320.1; 530/387.3, 388.22; 536/23.53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, CA, EMBASE, MEDLINE, WPI
search terms: P-selectin, PADGEM, GMP-140, paulson, polley, chesnut

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 56, issued 24 March 1989, G.J. Johnston et al., "Cloning of GMP-140, A Granule Membrane Protein of Platelets and Endothelium: Sequence Similarity to Proteins Involved in Cell Adhesion and Inflammation", pages 1033-1044, see entire document.	1-59
Y	Cell, Volume 59, issued 20 October 1989, E. Larsen et al., "PADGEM Protein: A Receptor That Mediates the Interaction of Activated Platelets with Neutrophils and Monocytes", pages 305-312, see entire document.	1-59
Y	Nature, Volume 343, issued 22 February 1990, J. Geng et al., "Rapid Neutrophil Adhesion to Activated Endothelium Mediated by GMP-140", pages 757-760, see entire document.	1-59

[X] Further documents are listed in the continuation of Box C.	[ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 JUNE 1994	0 1 AUG 1994

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer
	PHILLIP GAMBEL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Blood, Volume 77, Number 8, issued 15 April 1991, S. Parmentier et al., "Inhibition of Platelet Functions by a Monolconal Antibody (LYP20) Directed Against a Granule Membrane Glycoprotien (GMP-140/PADGEM)", pages 1734-1739, see entire document.	1-59
Y	J. Clin. Invest., Volume 90, issued October 1992, M.S. Mulligan et al., "Neutrophil-Dependent Acute Lung Injury", pages 1600-1607, see entire document.	1-59
Y	WO, A, 91/06632 (FURIE ET AL) 16 May 1991, see entire document.	1-59
Y	WO, A, 90/07861 (QUEEN ET AL) 26 July 1990, see entire document .	1-59

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.8; 435/69.6, 172.3, 252.3, 320.1; 530/387.3, 388.22; 536/23.53